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Study on the interaction of food supplements and enzymes involved in biotransformation of xenobiotics

Studium interakcí doplňků stravy s enzymy biotransformace xenobiotik

Diploma Thesis

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Prohlášení

Prohlašuji, že jsem tuto diplomovou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 15. 8. 2014 .....

podpis

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Abstract

Currently, an increasing attention is being paid to phytochemicals as one of the most widely used chemopreventive compounds, generally considered as health-promoting and safe. Flavonoids representing a large group of phytochemicals are present in many dietary supplements formulated from natural sources. The consumption of these concentrated phytochemicals has dramatically increased in the recent decade. It appears, however, that the ingestion of flavonoids might be associated with some adverse effects. Some flavonoids are known modulators of enzymes involved in phase I and phase II metabolism of xenobiotics biotransformation, thus their induction may result in an increase of carcinogen activation.

In this study, the effects of selected flavonoid compounds α-naphthoflavone, β-naphthoflavone, myricetin, and dihydromyricetin, and carcinogens (BaP, PhIP) on phase II metabolism enzymes, sulfotransferases (SULT), have been investigated. To determine the induction of SULT, antibodies for their immunodetection have been developed. Peptide antigens derived from sequences of selected rat sulfotransferases rSULT1A1, 1B1, 1C1, 1C2, 1C1/2, 1E1, and 2A1, were used as KLH conjugates for hen immunization to obtain yolk anti-peptide antibody (IgY). Fractions of IgY were isolated from eggs yolks by simple extraction and precipitation procedure with sodium chloride. The yield of IgY was about 150 mg per yolk (6.8 mg/ml of yolk). Since SULT1A1 is the most abundant SULT, the anti-peptide SULT1A1 IgY has been further purified by affinity chromatography in order to obtain only specific antibodies. However, even with affinity purified antibody, the effect of flavonoid compounds and carcinogens on SULT induction at the protein level was not possible to fully determine by immunodetection.

The activity of SULT in cytosol samples of animals treated with flavonoids or carcinogens has been studied as well. In this study, two assays of SULT enzymatic activity were tested. Being aware that the tissue cytosol samples are usually a complex mixture of different isoenzymes of SULT, we optimized the determination of the activity based on p-nitrophenyl sulfate detection on HPLC. SULT activities in rat liver cytosols, with the exception of PhIP administration, did not show marked changes in response to animal treatment, when compared with the SULT activity of un-treated animals. The SULT activities in colon cytosols were approximately 100x lower in comparison to liver cytosols and did not significantly change after animal treatment.

key-words: sulfotransferases, flavonoids, chicken antibodies

Abstrakt

V poslední době se věnuje stále větší pozornost chemopreventivním sloučeninám a to zejména fytochemikáliím, které jsou všeobecně považovány za zdraví prospěšné látky a to hlavně díky jejich přírodnímu původu. Velkou skupinu fytochemikálií představují flavonoidy, které jsou obsaženy v mnoha potravních doplňcích. Spotřeba těchto koncentrovaných zdrojů flavonoidů se v posledních letech dramaticky zvýšila. Užívání flavonoidních látek ve velkém množství však může být spojeno s některými nežádoucími účinky. Některé flavonoidy jsou známé modulátory enzymů I. a II. fáze biotransformace xenobiotik a jejich indukce může vést ke zvýšení aktivace karcinogenů.

V rámci této diplomové práce byly zkoumány účinky vybraných flavonoidních sloučenin α-naftoflavonu, β-naftoflavonu, myricetinu a dihydromyricetinu a karcinogenů (BaP, PhIP) na enzymy II. fáze biotransformace, sulfotransferasy (SULT). Pro stanovení indukčního efektu byly připraveny protilátky pro jejich imunodetekci. Peptidické imunogeny, které byly vybrány z proteinových sekvencí potkaních sulfotransferas rSULT1A1, 1B1, 1C1, 1C2, 1C1/2, 1E1 a 2A1 byly použity ve formě KLH konjugátů pro imunizaci slepic za účelem získání antipeptidových protilátek (IgY) z vaječných žloutků. Frakce jednotlivých IgY byly izolovány jednoduchou extrakcí a následným vysrážením pomocí chloridu sodného. Výtěžek IgY činil asi 150 mg/žloutek (6,8 mg/ml žloutku). Mezi nejhojnější enzymy patří SULT1A1, proto byla protilátková frakce proti tomuto proteinu dále purifikována pomocí afinitní chromatografie, čímž byly získány pouze specifické protilátky. Účinek flavonoidních látek a karcinogenů na indukci SULT na úrovni proteinu však nebylo možné detekovat pomocí metody „Western blot“, a to ani s afinitně purifikovanou protilátkou SULT1A1.

Následně byla v cytosolárních vzorcích z premedikovaných zvířat zkoumána aktivita SULT. V rámci této práce byly testovány dvě metody stanovení enzymové aktivity sulfotransferas. S ohledem na to, že v cytosolárních vzorcích z tkáně potkanů je obvykle přítomno více isoenzymů SULT, byla optimalizována metoda stanovení aktivity SULT na základě detekce sulfatovaného p-nitrofenolu pomocí HPLC. Aktivity sulfotransferas v potkaních jaterních cytosolech premedikovaných zvířat, s výjimkou PhIP, se výrazně nezměnily. Aktivity sulfotransferas v cytosolech získaných z tlustého střeva premedikovaných potkanů byly až 100x nižší v porovnání s jaterní tkání a nevykázaly výrazné změny v závislosti na premedikaci.

klíčová slova: sulfotransferasy, flavonoidy, slepičí protilátky

Contents

List of abbreviations

AαC	2-amino-9H-pyrido[2,3-b]indole
ADP	adenosine diphosphate
AIA	aminoimidazoarenes
ANF	α-naphthoflavone
AUC	area under the curve
BaP	benzo[*a*]pyrene
BCA	bicinchoninic acid
BCIP/NTB	5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tab.
BNF	β-naphthoflavone
BSA	bovine serum albumin
b.w	body weight
CYP	cytochrome P450
DEA	diethylamine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
GST	glutathione S-transferase
HAA	heterocyclic aromatic amines
CHST	carbohydrate sulfotransferases
IARC	International Agency for Research on Cancer
IgG	immunoglobulin (antibody) of class G
IgY	chicken antibody
IQ	2-Amino-3-methylimidazo[4,5-f]quinoline
kDa	kilodalton
mcKLH	maleimide activated keyhole limpet hemocyanin
MeAαC	3-methyl-9H-pyrido[2,3-b]indole
MeIQ	2-Amino-3,4-dimethylimidazo[4,5-f]quinoline
MFO	mixed function oxidise
mRNA	messenger RNA
NAT	N-acetyltransferase

N-OH-PhIP	N-hydroxylated PhIP
O/N	overnight
PAP	3'-phosphoadenosine-5'-phosphate
PAPS	3'-phosphoadenosine-5'-phosphosulfate
PBS	phosphate buffer saline
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
pNP	p-nitrophenol
pNPP	p-nitrophenyl phosphate
pNPS	p-nitrophenyl sulfate
ROS	reactive oxygen species
RP-HPLC	reverse phase high-performance liquid chromatography
RPM	revolutions per minute
SD	standard deviation
SDS	sodium dodecyl sulfate
SNP	single nucleotide polymorfism
ST	sulfotransferase
SULT	cytosolic sulfotransferase
TPST	tyrosyl protein sulfotransferases
TRIS	tris(hydroxymethyl)aminomethane
UGT	UDP- glucuronosyltransferase
UV	ultra violet
v/v	volume/volume percent
w/v	weight/volume percent

1. INTRODUCTION

1.1. Biotransformation of xenobiotics

Organisms are constantly exposed to external physical, biological, and chemical influences that may affect the natural physiological processes, including metabolism. Many of the chemical exogenous compounds (xenobiotics) are potentially harmful, hence, the human body has developed an effective system of biotransformation of such foreign compounds. The process of biotransformation is defined as an enzyme-catalysed conversion of mostly lipophilic compounds into other derivatives, which are in their final form polar molecules, and may be easily excreted from the body. The principal biotransformation organ is the liver, where biotransformation enzymes are mostly located. The other organs participating in these processes are the kidneys, lungs, small intestine, colon, epithelial cells, and skin.

Xenobiotics mainly include environmental pollutants, drugs, food additives, toxins, and carcinogens. They may interact with organism in 3 different ways: (i) a compound does not interact with an organism at all and it is excreted in an unchanged form, (ii) it is accumulated in a tissue (mainly an adipose tissue), or, (iii) a compound undergoes phase I and/or phase II metabolism (= biotransformation) in order to be processed for excretion.

1.1.1. Phase I metabolism

During phase I, polar groups such as -OH, -COOH, and -NH_2 are uncovered or incorporated into chemicals in order to form more hydrophilic compounds. However, depending on the type of compound (drugs, hormones, bile acids, peptides, chemical carcinogens etc.) the result of this process may vary:

- → Initially hydrophobic endobiotics become more easily excretable and are excreted in urine and bile,
- → Pharmacologically active compounds or xenobiotics are inactivated and subsequently excreted as well,
- → Initially less harmful xenobiotic intermediates can be metabolically activated into compounds with higher toxicity or carcinogenic potential.

This phase is characterized by oxidation, hydroxylation, dealkylation, reduction and hydrolytic reactions, which occur mainly in endoplasmic reticulum. Major enzymes involved in phase I are flavin-containing monooxygenases, peroxidases, and cytochromes P450.

The most important reaction is oxidation, especially through so called *Mixed Function Oxidase* (MFO) system. MFO represents a group of enzymes, catalysing oxidation, oxygenation, or reduction of compounds. The key enzymes involved in these reactions are cytochromes P450 (CYPs), which play a significant role in the metabolism of xenobiotics as well as hydrophobic endogenous substrates. The term refers to the whole group of membrane hemoproteins, which serve as terminal oxidases and possess noncovalently bound protoporphyrin IX [1].

1.1.2. Phase II metabolism

Either already suitable polar group containing intermediates originating from phase I, or incoming relatively polar endogenous/xenobiotic compounds enter phase II of biotransformation in order to further conjugate with hydrophilic endogenous molecules. As well as in the Phase I metabolism, the results of these processes are not uniform. The addition of large anionic groups detoxifies reactive electrophiles and produces more polar metabolites that cannot diffuse across the membranes, and may, therefore, be actively transported. On the other hand, even more polar metabolites with a hydrophilic function group can be unstable and thus degrade into compounds with higher toxicity and carcinogenic potential.

A number of enzyme systems contributing to phase II metabolism are found in the endoplasmic reticulum and in the cytosol of the cell. In mammals, as mentioned, they are encountered in every tissue, but particularly in the liver, the main detoxification organ [2]. Phase II metabolising enzymes are a large group of broad-specificity transferases: UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), N-acetyltransferases (NATs), glutathione S-transferases (GSTs), and various methyltransferases.

Individual conjugation reactions with the appropriate participating enzymes are discussed in the following sections.

1.1.2.1. *Sulfation*

Sulfation is the enzyme-catalysed addition of a sulfuryl group from a donor molecule, 3'- phosphoadenosine - 5'-phosphosulfate (PAPS) to a variety of amine and hydroxylated substrates. Sulfation is catalysed by a group of enzymes called sulfotransferases (STs). In mammals, there are two classes of sulfotransferases: membrane-associated sulfotransferases and cytosolic sulfotransferases.

Membrane-associated sulfotransferases (STs) catalyse sulfation of larger biomolecules, such as carbohydrates and proteins, and are located in the membranes of the Golgi apparatus. In fact, STs do not participate in biotransformation of xenobiotics. However, despite the difference in location, STs also use PAPS as a sulfate donor, which is delivered to the Golgi apparatus by specific transporters [3]. Their biological function is mostly in signal transduction processes, leukocyte adhesion, and anticoagulation events [4]. Examples include a family of tyrosyl protein sulfotransferases (TPSTs), which participate in posttranslational modifications of proteins, or a family of carbohydrate sulfotransferases (CHSTs) that play a crucial role in extracellular signaling and adhesion by generating unique ligands from a carbohydrate scaffold [5,6]. A consistent nomenclature still lacks for STs, therefore they are usually named according to their substrate specificity, using numbers to differ isoenzymes (e.g. carbohydrate sulfotransferase 1, CHST-1).

A class of cytosolic sulfotransferases significantly participates in phase II metabolism. Since the cytosolic sulfation is the main subject of this Master Thesis, a special attention to them is devoted in a Chapter 1.2.

1.1.2.2. *Conjugation with acetyl moiety*

Another important conjugation reaction is the transfer of the acetyl group from acetyl-CoA to hydrophobic arylamin to produce arylacetamide. The reaction occurs as two-step reaction: first, acetyl is transferred to a cysteine residue of the enzyme forming an acetyl-cysteinyl-enzyme catalytic intermediate, and second, the acetyl is transferred to the amino nitrogen of an acceptor molecule. Enzymes, which catalyse this detoxication *N*-acetylation are called arylamine N-acetyltransferases (NATs). In addition to *N*-acetylation, they are also capable of catalysing *O*-acetylation, when the acyl group is transferred into the hydroxyl group of arylhydroxyamines resulting in acetoxyamine intermediates.

Acetoxyamines are, however, unstable intermediates that disintegrate into mutagenic compounds [2].

N-acetyltransferases are cytosolic enzymes widely distributed across species and throughout many tissues. In humans, there are two genes expressing two isoenzymes NAT1 and NAT2. Both isoenzymes acetylate a range of xenobiotic compounds such as arylamines and heterocyclic aromatic amines, as well as various therapeutic agents and drugs. The nucleotide sequences of these two genes show 85% homology but differ in substrate specificity and tissue distribution. NAT1 has no preferences in distribution while NAT2 activity has been detected mostly in liver, colon, and intestinal epithelium [7]. Typical substrate for hNAT1 is the p-aminobenzoic acid, and sulfamethazine for NAT2, respectively. Interestingly, to date, no role of NAT in endogenous metabolism was found.

A prominent feature of these enzymes is their genetic polymorphism. Individual differences in the NATs metabolic capacity are caused by allelic variations of the NATs gene which are determined by a single nucleotide polymorfism (SNP) resulting in rapid, intermediate, or slow acetylator phenotypes [8].

Rat arylamine N-acetyltransferases are very similar in sequence and function with human N-acetyltransferases. However, there are some interspecies differences. For example, p-aminobenzoic acid, a selective substrate for hNAT1, is a selective substrate for rNAT2. In addition, new rat gene *Nat3* has been described and characterized recently [9]. Amino acid sequence rNAT3 is about 67% identical to human NAT1 and NAT2, respectively.

1.1.2.3. *Glucuronidation*

The formation of glucuronide conjugates is the most important detoxication pathway in all vertebrates [7]. Moreover, approximately 40-70% of all clinical drugs are metabolized through this metabolic pathway in humans [10].

Glucuronidation is catalysed by UDP-glucuronosyltransferases (UGTs), internal membrane-bound enzymes with receptors exposed to the lumen of endoplasmic reticulum. This provides an advantage that these enzymes have direct access to metabolites produced by phase I metabolism [11]. The mechanisms of catalysis include the transfer of glycosyl group of a nucleotide sugar to an acceptor compound (intermediate) at a nucleophilic functional group of oxygen (e.g. hydroxyl or carboxylic acid groups), nitrogen (e.g.

amines), sulphur (e.g. thiols), and carbon with the formation of a final β-D-glucuronide product. β-D-glucuronides are then excreted by bile or urine.

UGTs differ in occurrence among organs as well as among individuals. To date, 80 families containing over 850 UGTs with diverse substrate specificities have been identified in animals, plants, and microorganisms [12]. Humans have four UGT families: UGT1, UGT2 (divided into two subfamilies 2A and 2B), UGT3 and UGT8. UGT1 family has nine members, mostly participating on glucuronidation of bilirubin, estrogens, bile acids, drugs, and xenobiotics. The UGT2 family contains three members of the UGT2A subfamily and seven members of the UGT2B subfamily. Many of them, but not all, are located mainly in the liver tissue. Among the members with the highest importance in drug metabolism belong UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7 and 2B15. This is mainly because of the influx of drugs from the hepatic portal vein after oral ingestion. Some isoenzymes located in the gastrointestinal tract also have particular importance, since they participate in dietary supplements and drug metabolism [7].

In contrast to the previously mentioned families, UGT8 family has only one member that plays a biosynthetic role in the nervous system by participating in glycosfingolipids and cerebrosides formation [13]. Until recently, catalytic function of the last family, UGT3, has remained unknown. One of the two members, UGT3A1, was identified as a UDP N-acetylglucosaminyltransferase [12].

1.1.2.4. *Conjugation with glutathione*

Glutathione is a tripeptidic compound (γ-Glu-Cys-Gly) with high antioxidant capacity, preventing damage of cells caused by free radicals and other reactive molecules. In addition to that and other minor functions, it also serves as a conjugation partner in the detoxication processes of endogenous and exogenous metabolites. The conjugation of glutathione *via* its sulphur atom with electrophilic molecules is catalysed by enzymes called glutathione S-transferases (GST).

There are 3 major families of proteins with glutathione transferase activity. The cytosolic and mitochondrial GSTs include dimeric soluble enzymes that are involved in the biotransformation of toxic xenobiotics and endobiotics. The third family, a group of microsomal GST, is referred to as membrane-associated proteins in eicosanoid and glutathione metabolism and are structurally distinct from the previous two families [14,15].

The cytosolic and mitochondrial GSTs are represented jointly by 8 classes: alpha (α), mu (μ), pi (π), zeta (ξ), theta (τ), sigma (σ), kappa (κ), and omega (ω). Only five classes have their orthologs in humans.

All three families are widely distributed throughout the body and their major locations include the tissue of liver, kidney, brain, pancreas, lung, and small intestine [16].

1.1.2.5. *Methylation*

Unlike the other phase II detoxication processes, methylation has only small importance in the metabolism of xenobiotics. However, some anticancer and immunosuppressive drugs are being inactivated to excretable methylated metabolites by this pathway [17]. There are two types of methylation: *O*-methylation and *N*-methylation. During both reactions, the methyl group from S-adenosyl-L-methionine is conjugated to reactive hydroxy or amine group on an intermediate metabolite.

1.1.2.6. *Amino acid conjugation*

Some cyclic and aromatic amino acids are not degraded by means of β-oxidation but rather conjugate with other amino acid to produce more soluble molecules. Firstly, the amino acids are activated by acyl-CoA-synthetases to acyl-CoA and secondly, they are conjugated with another amino acid, mostly with glycine, taurine or glutamic acid. Those processes occur in mitochondrial matrix [18].

1.2. Cytosolic sulfotransferases

Sulfation, but more correctly referred to as sulfonation or sulfurylation, is an important phase II conjugation reaction, which is catalysed by the supergene family of enzymes called cytosolic sulfotransferases (SULTs). These enzymes specifically catalyse the transfer of the sulfuryl group (SO_3^-; sometimes called sulfate) from an endogenous sulfate donor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) into a variety of acceptor substrates. These substrates include a wide range of xenobiotics and drugs as well as endogenous molecules (e.g. hormones, steroids, and vitamins). In general, *O*-sulfation represents the dominant reaction, however, *N*-sulfation is a crucial way of modification of carbohydrate chains in macromolecules such as heparin, a common component of proteoglycans [19]. SULTs have a significant role in a number of biological processes including drug processing, xenobiotics detoxication, molecular recognition, hormone regulation, and modulation of receptor binding [20].

1.2.1. Classification and nomenclature of sulfotransferases

Nomenclature of cytosolic sulfotransferases has not been always unified and the currently used new nomenclature system was established in 2003 by Blanchard [21]. Nevertheless, many different names for the same enzyme can still be encountered in the literature, however, the new actual nomenclature guidelines are followed in this thesis.

All cytosolic sulfotransferases are members of a single supergene family, termed SULT. They can be divided into several families and subfamilies according to their sequence identity. The members of the same gene family share 45% sequence homology, while members of each subfamily share even 60% and more identity. The classification thus requires rules for identification:

- Families are designated by a number (e.g. SULT1).
- Subfamilies are identified by a capital letter after the family number (e.g. SULT1A).
- Unique isoforms are identified by a number following the subfamily letter (e.g. SULT1A1).

- Allelic protein variants are identified by an asterisk and a number following the gene isoform number suffixed to the isoform of protein name (e.g. SULT2A1*1A).
- Isoforms with different amino acid sequences that are, however, encoded by an identical gene are differentiated by "_vx" at the end, where "x" stands for sequential variant number. For example, the proteins initially referred to as SULT2B1a and SULT2B1b are named SULT2B1_v1 and SULT2B1_v2, respectively.
- Species names are designated by letters prefixed to the SULT name (e.g rSULT = *rat*, hSULT = *human*, mSULT = *mouse*).

Enzymes vary in their structures, substrate specificities and enzyme localization between species. In terms of carcinogen metabolism research, hSULT and rSULT are enzymes of interest, since *in vivo* experiments with rat models provide relevant information because of high similarity with the human ones.

To date, 4 human families have been identified containing 13 genes encoding hSULTs:

hSULT1

Family hSULT1 involves 8 members and is divided into 4 subfamilies **A**, **B**, **C**, and **E**: **1A1**, **1A2**, **1A3/4**, **1B1**, **1C2**, **1C3**, **1C4**, and **1E1**. In fact, there are two genes *1A3* and *1A4*, which are almost duplicates, thus encode an identical protein 1A3/4. This family is primary involved in metabolism of endogenous steroid compounds and xenobiotics, therefore it is sometimes called as the family of phenolsulfotransferases.

hSULT2

Family hSULT2 contains sulfotransferases, which are subdivided into 2 subfamiles with 3 members: **2A1**, **2B1_v1**, and **2B1_v2**. 2B1 isoforms encoded by a single gene only distinct in 15 amino acids at the amino terminus, although they significantly differ in substrate specificity. Since the substrate specificity is towards hydroxysteroids, they are also called hydroxysteroid sulfotransferases.

hSULT4

Family hSULT4 is represented only by an orphan **4A1**. No known substrate or function has been confidently identified for the hSULT4 family yet.

hSULT6

Family hSULT6 is also represented by an orphan **6A1**. In humans, it has neither the protein nor its enzymatic activity characterized as well.

Classification of rSULT is similar to hSULT. There are 3 families containing 13 enzymes:

rSULT1

rSULT1 family includes several members: **1A1**, **1B1**, **1C1**, **1C2**, **1C2A**, **1D1**, and **1E1**, which show substrate specificity towards phenolic compounds, like in humans.

rSULT2

rSULT2 family includes 5 members: **2A1**, **2A2**, **2A3**, **2B1_v1**,and **2B1_v2**.

rSULT4

This family, as well as the human one, includes only an orphan member, so called "sult-like protein" **4A1**.

1.2.2. Tissue distribution

The expression of SULTs can be studied at various levels - enzyme activity or protein (Western blot) or mRNA level. Each method has its limitations in specificity and accuracy. Furthermore, a high level of mRNA is not always associated with a high level of protein expression and enzyme activity, thus the data are difficult to compare. There are also inter-individual differences in SULT expression in the population and many allelic variants with differential kinetic profiles have been identified.

The tissue distribution of five principal hSULTs is the most extensively studied: 1A1, 1B1, 1A3/4, 1E1 and 2A1. Based on quantitative immunoblotting, hSULT1A1 is the major enzyme in the liver (relative contribution 53%), followed by hSULT2A1, hSULT1B1, and hSULT1E1, as well as 1A1 is the major enzyme in the kidney [22]. Small

intestine is the major site of expression for hSULT1B1, followed by hSULT1A3 and hSULT1A1. Several orphan hSULT are found only in specific organs, e.g. hSULT4A1 was detected only in neurons located in several regions of the brain. In addition, hSULT4A1 has little sequence homology with other human SULTs (40%) and yet it is highly conserved between species (98%) [23]. Interestingly, expression of the SULT1C subfamily is found predominantly in the human fetus (fetal kidney, lung, heart, and GIT) [7].

The tissue distribution of SULTs strongly differs in humans and in other species. In rat, SULTs are expressed mainly in the liver. In addition, enzymes are mostly sex-specific, e.g. rSULT1C1 is almost exclusively expressed in males. For instance, Northern blot analysis showed that male dominant SULT expression of SULT1A1 was observed in different proportions in liver (major expression) and additionally also in the lung, brain, heart, intestine, kidney, testes, spleen, and adrenal tissue while another major enzyme SULT1B1 was detected only in liver, intestine, and kidney [24].

1.2.3. Structure of SULTs and mechanism of their catalysis

SULTs are single α/β globular proteins with a characteristic four- or five-stranded parallel β-sheets surrounded by α-helices (Fig.1). PAPS-binding site together with catalytic site is constituted by β-strands, however, while the PAPS binding activity is common among the various SULTs, the substrate binding activity is unique for individual ones, indicating significant structural variation of the catalytic centre [20].

The kinetic mechanism has been unclear for a long time, but all the reactions studied with SULTs from higher organisms proceed by sequential mechanisms, which are either random or ordered. The mechanism is also called “bi-bi”, since the reaction involves two substrates, that lead to two products [4].

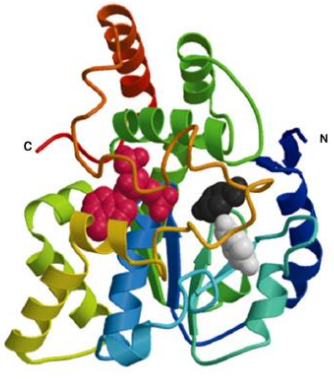


Figure 1: Crystal structure of human SULT1A1 complexed with 3’- phosphoadenosine - 5’-phosphate (PAP; dark pink) **and two p-nitrophenol molecules** (dark grey and white). *Adapted from* [25].

1.2.4. SULTs and bioactivation of xenobiotics

In case of xenobiotics, sulfation is considered to be a detoxication process that generates less or non-toxic metabolites. However, some compounds with procarcinogenic and promutagenic potential can be metabolically activated into unstable sulfate conjugates, which after -SO_3 cleavage become reactive and can cause damage to DNA by covalently binding to its bases. Such reactivity is primary caused by the sulfate anion, whose sulphur has an electron-withdrawing character, leading to the formation of nitrenium cations (Fig.2). This effect will only occur when the heterolytic cleavage of the sulfate group is facilitated (such cleavage is possible if the resulting cation is stabilized by inductive effects or by mesomerism). As a result, strongly electrophilic potential of nitrenium ion (carbonium ion, eventually) drives the reactions with nucleophilic molecules, including proteins and DNA, forming adducts and resulting in mutation [26].

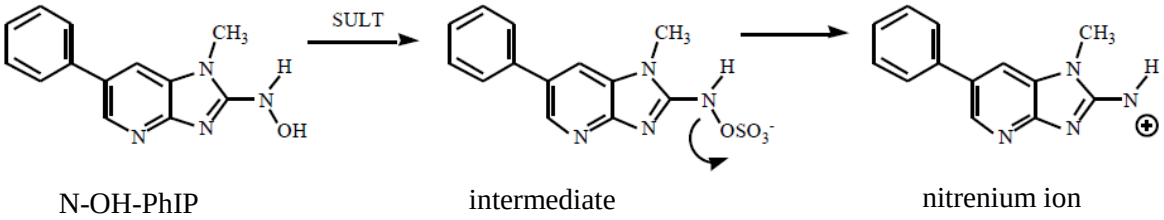


Figure 2: Formation of reactive electrophilic nitrenium ion from procarcinogenic N-hydroxylated 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (N-OH-PhIP). *Adapted from* [20].

1.2.5. Inhibition and induction of SULTs

An important feature of enzyme catalysis is its regulation, which may be dual: regulation at the level of gene expression and regulation of enzyme activity at the protein level. Enzyme activity can be regulated by the so called modulators (activators or inhibitors, respectively). Irreversible modulation occurs if the modulators are covalently bound to the enzyme, otherwise, it is a reversible process [27]. The quantity of the enzyme at gene expression level as well as the enzyme activity can be also regulated to a certain extent. While the expression level of the constitutive enzymes cannot be influenced by means of presence or absence of a specific stimulus, inducible enzymes are sensitive to exogenous inductors/repressors and upregulation/downregulation may occur.

Inducibility, as a significant feature of enzymes, plays a major role in the biotransformation processes. Repressors and inductors are mostly drugs, food additives and food supplements, especially phytochemicals. There are several known flavonoid modulators of sulfotransferases, which have been determined in experiments *in vitro* and *in vivo*. In general, inhibition of carcinogen-metabolizing enzymes is considered to lead to the decrease in the level of mutagenic molecules and therefore to chemopreventive effects. The inhibitors of SULTs include e.g. kaempferol, chrysin, apigenin or genistein [28]. More importantly, induction of SULT enzymes by natural compounds has been also proven even in *in vivo* studies [29-31]. As already indicated, some dietary procarcinogens are metabolically activated by SULTs into reactive carcinogens. Such induction could thus lead to the potentiation of metabolic activation. The inductors of SULTs include compounds such as caffeine or biochanin A [30,31].

For Chemoprevention see Chapter 1.4.

1.2.6. The most abundant enzyme SULT1A1

Sulfotransferase 1A1 is the enzyme of interest of this Thesis. It is the most widely studied SULT in detail, due to its broad substrate specificity, wide tissue distribution, and involvement in the metabolism of xenobiotics including the bioactivation of dietary procarcinogens [32]. It was originally referred to as the aryl/phenol or thermostable sulfotransferase (P-PST or TS-PST).

The form hSULT1A1 is responsible for the sulfoconjugation of steroid endogenous compounds, e.g. β-estradiol; thyroid hormones; dopamine; xenobiotics, e.g. p-nitrophenol, p-naphthol, hydroxylamines, heterocyclic aromatic amines such as 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP); and drugs, e.g. hydroxytamoxifen, Minoxidil etc. [7,33,34]. 7 allelic variants of hSULT1A1 have been identified, of which the most common ones are hSULT1A1*1 a hSULT1A1*2. The first mentioned exhibits higher activity towards substrates along with higher thermostability and higher bioactivation potencial. In fact, hSULT1A1 is now known to activate various procarcinogens, such as hydroxymethyl polycyclic aromatic hydrocarbons, N-hydroxy derivates of arylamines, heterocyclic amines, into highly reactive intermediates [35]. Rat ortholog, rSULT1A1, has a very similar substrate specificity and tissue distribution as a human enzyme, since they share 80% homology.

1.3. Chemical carcinogenesis

Cancer is currently one of the leading diseases in human population with one of the highest mortalities. The process of developing cancer, carcinogenesis, is influenced by many factors and much progress has been made in the understanding of its mechanism and development in the last 50 years. It is believed, that over 80% of cancer deaths may be attributed to factors such as alcohol, tobacco, diet, and environmental factors [36]. Those factors represent the so called chemical carcinogenesis, since the carcinogens are particular chemical compounds. Despite the chemical and structural diversity of these chemicals, the mechanisms of tumorigenesis are fundamentally similar. The active metabolites of most carcinogens are electrophiles (or reactive oxygen species) that react with nucleophilic DNA inducing mutations and/or genotoxic changes [37].

The carcinogenesis is a multilevel process, which consists of 3 stages: initiation, promotion, and progression. Tumour initiation begins when DNA in cell is damaged by an exposure to carcinogenic compounds leading to the mutation in critical target genes. Next, the tumour promotion stage is characterized by a selective clonal expansion of the initiated cells, caused by altered expression of proto-oncogenes and tumour suppressor genes. Finally, the progression stage is characterized by malignant transformation of preneoplastic cells. However, the carcinogenesis is rather complex process than strictly divided, since the genetic background and nutritional status can dramatically affect susceptibility to a carcinogenetic exposure [38].

1.3.1. Carcinogens

Carcinogens can be classified into two groups based on their ability to act as mutagenic agents.

First group, genotoxic carcinogens, are able to irreversibly alter genetic information of the cell (DNA). In high doses, they cause cell proliferation, increasing DNA replication and influence its carcinogenic activity [39]. Following the transmembrane diffusion, they are metabolized into electrophilic reactive compounds that enter the nucleus and interact with nucleophilic molecules (DNA, RNA, and proteins), changing their structural integrity and establishing covalent bonds known as adducts. Most serious damage is caused when the mutated genes are involved in signaling, cell cycle regulation, and apoptosis.

On the other hand, epigenetic carcinogens serve as so called co-carcinogens. Such compounds directly potentiate carcinogen's mutagenicity [40], but themselves show negative results on mutagenicity tests *in vivo* and *in vitro*. They do not react with genetic information, but rather modulate growth and cell death, in dependence on their concentration. Most of the epigenetic co-carcinogens are hormones, largely synthetic pesticides, food additives, and medicines [41].

According to International Agency for Research on Cancer (IARC), carcinogens and potential carcinogens are divided into 5 groups (Tab. 1).

Table 1: IARC classification of carcinogens *(examples included)* [42]

Group 1	***Carcinogenic* to humans** e.g. benzo[*a*]pyrene
Group 2A	***Probably carcinogenic* to humans** 2-Amino-3-methylimidazo[4,5-f]quinoline (IQ)
Group 2B	***Possibly carcinogenic* to humans** 2-Amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ), 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)
Group 3	***Not classifiable as to its carcinogenicity* to humans** phenol
Group 4	***Probably not carcinogenic* to humans** caprolactam

Note: To date, the database includes 970 chemicals.

In addition to the individual effects of chemical carcinogens, synergic or antagonistic effects may be observed. For instance, the synergism between smoking and asbestos exposure results in a highly probable lung cancer development as a consequence of chronic inflammation together with regenerative cell proliferation at the site of fibre deposition. The antagonism, on the other hand, is represented by cancer preventive factors such a fruit and vegetable consumption, which may result in carcinogenesis suppression [43].

Some carcinogenic compounds, such as aflatoxines, polycyclic aromatic hydrocarbons, and heterocyclic aromatic amines are present in the diet. They may occur in the diet naturally, as contaminants, or arise during food processing or heating. Food carcinogens are usually formed in very small quantities so that they do not pose an acute

risk. However, effects of all uptakes of carcinogens over a longer period may result in carcinogenesis.

Since the food carcinogens, especially heterocyclic aromatic amines, are those of interest, they are briefly characterized in the following section.

1.3.1.1. *Heterocyclic aromatic amines*

Heterocyclic aromatic amines (HAAs) are a class of pro-carcinogenic compounds that are formed during high temperature cooking of meats including fish, especially during the processes of frying and grilling [44]. In addition, some of them are also present in the cigarette smoke [45] as well as in airborne particles generated by grilling of meats [46].

More than 20 HAAs have been identified and they can be divided into two groups according to how they arise: pyrolytic HAAs and aminoimidazoarenes (AIA). Pyrolytic amines arise during the pyrolysis (> 250°C) of individual amino acids such as tryptophan, glutamic acid, and phenylalanine, or during the high-temperature heating of proteins. In fact, high temperature catalyses the formation of deaminated and decarboxylated amino acids and reactive fragments, which then combine to form heterocyclic structures [44]. The most abundant HAAs of this group are 2-amino-9H-pyrido[2,3-b]indole (AαC), 2-amino-3-methyl-9H-pyrido[2,3-b]indole (MeAαC), and 2-amino-6-methyldipyrido[1,2-a:3′,2′-d]imidazole (Glu-P-1). The second class of HAAs is formed during meat heating in temperatures between 150-250°C, representing the commonly utilized temperature range in household kitchens. Amino acids, sugars, and creatine are precursors to these HAAs. The 2-amino-N-methylimidazole part of the HAA molecule is derived from creatine, and the remaining parts of the skeleton are assumed to arise from products formed in the Maillard reaction between sugars and amino acids [47]. The most abundant members are 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx).

The quantity of HAAs formed depends on the type of meat and the method of cooking. Generally, frying and grilling of meats produce the largest concentration of HAAs, whereas the roasting and broiling of meats generate significantly smaller quantities. Stewing, boiling and microwave cooking are thought to be the mildest forms of meat preparation. For instance, the content of HAAs in poultry products, in dependence on type of heat-treatment has been reviewed [48].

2-Amino-1-methyl-6-fenylimidazo[4,5-b]pyridine

2-Amino-1-methyl-6-fenylimidazo[4,5-b]pyridine (PhIP) is one of the most abundant AIA formed in meat cooked using high-temperature methods. In order to understand the underlying mechanisms of the formation of PhIP, controlled model systems were utilized. It was shown that PhIP consists of a mixture of compounds including creatinine, phenylalanine, sugars, and aldehydes [49]. Although PhIP can be directly excreted from the body after consumption, it is also extensively metabolized into a variety of metabolites. Once PhIP is absorbed from the gastrointestinal tract, it is principally metabolized in the liver. PhIP is a procarcinogen so it requires an activation step in order to exert its carcinogenic potency. Enzymes participating in metabolic activation in phase I are CYPs (primary CYP1A2), generating the intermediate metabolite N^2-hydroxy PhIP, which is further esterified by NAT2 and SULT1A1 to N^2-acetoxy PhIP and N^2-sulfonyloxy PhIP [50,51]. These very reactive intermediates are known to create DNA adducts *via* electrophilic nitrenium ion by its binding to the guanine base on DNA. However, conjugations by phase II enzymes UGT1A1 and GSTs can usually lead to the production of safe metabolites and efficient excretion of PhIP [44,52]. Overview of major biotransformation pathways of PhIP in human and rat hepatocytes are depicted in Fig. 3.

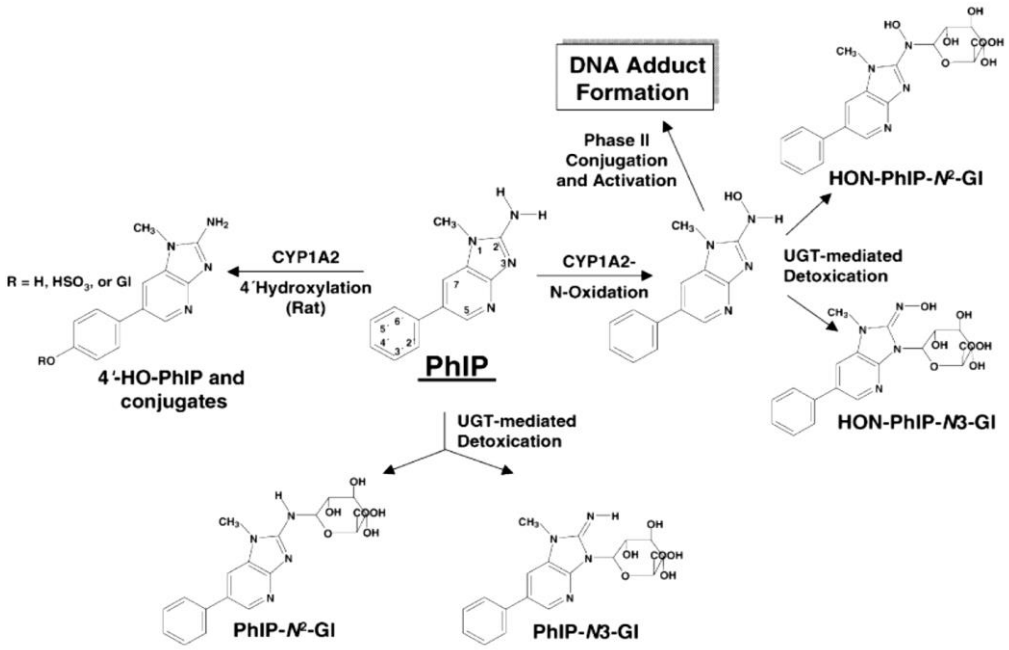


Figure 3: Biotransformation of PhIP. *In humans, PhIP undergoes either detoxication process via UGT-mediated metabolic pathway, or N-oxidation via CYP1A2. In rat, PhIP is always hydroxylated via CYP1A2 and then acetylated or conjugated with sulfate. Adapted from* [53].

Intra-species differences between humans and rat models in PhIP metabolism were found. For instance, a larger proportion of N-hydroxylated metabolites was found in human urine and plasma when compared to those of rodents. Further, it was shown that humans possess lower capacity for detoxification processes relative to rodents, suggesting that rodent models do not accurately represent the human response to heterocyclic amine and the data can not be always directly compared [53,54].

1.4. Chemoprevention

Chemoprevention comprises multiple intervention methods using either pharmacological or dietary agents to impede, arrest, or reverse carcinogenesis at its stages - initiation, promotion and progression (Fig. 4) [55].

According to the endpoints and potential targets, most chemopreventive compounds can be classified as either blocking or suppressing agents. Blocking agents aim to prevent the occurrence of DNA mutation by carcinogens (initiation phase) and are represented mainly by substances capable of metabolic activation-inhibiting, free radicals scavenging or carcinogen trapping. On the other side, suppressing agents mostly interfere with the promotion and progression phase of carcinogenesis. They influence cell proliferation, differentiation, and apoptosis [55].

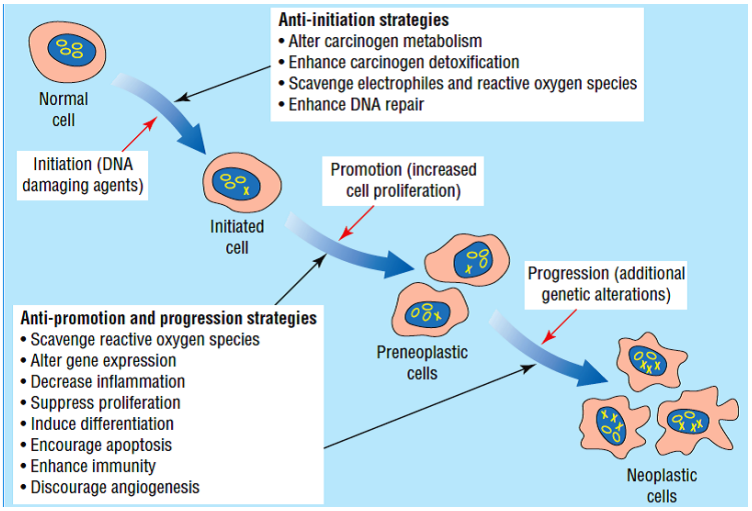


Figure 4: Processes and chemoprevention strategies. *Cancer is a multistage disease and may be affected by chemopreventive compounds by means of blocking or suppression. Initiation phase of carcinogenesis may be blocked by several anti-initiation strategies, while promotion and progression phases may be suppressed by several events. Adapted from [56].*

There are several classes of chemopreventive compounds: carotenoids, vitamins, phenolic compounds, flavonoids, isothiocyanates etc. It is their natural origin, which attracts potential consumers of dietary supplements. On the other hand, they have to be perceived as xenobiotics to humans, so their long-term administration may exhibit adverse effects. Their negative activities result namely from:

- their toxicity *per se*,
- metabolic conversion into cytotoxic, pro-oxidant or mutagenic agents,
- interference with endogenous metabolic pathways,
- interaction with other chemicals from diet, environment, or drugs,
- induction of carcinogen activating enzymes,
- effects on human intestinal microflora [57].

1.4.1. Flavonoids

Flavonoids are one of the most abundant phenolic compounds, which are present in fruits, vegetables, herbs, and beverages such as wine, tea, and coffee. In general, flavonoids are a class of plant secondary metabolites, which fulfil many functions including floral pigmentation, pathogen protection, chemical messaging or physiological regulations.

The basic flavonoid structure is formed by a heterocyclic flavone backbone, and based on the differences in structure and functional groups present, they are classified into 6 subclasses: flavonols, flavones, flavanols (catechins), flavanones, anthocyanins, and isoflavones [58]. However, flavonoids naturally occur as glycosides (aglycone + sugar moiety attached) and their structure is usually more complex - there are about 80 different sugars identified to bind flavonoids [59]. Since the chemical structure and some properties of several flavonoids are similar to those of naturally occurring estrogens, they are also called phytoestrogens (especially isoflavones).

1.4.2. Biological activities of flavonoids

A variety of health-promoting properties are attributed to flavonoids. The most important ones include: antioxidant potential, scavenging of free radicals, anti-tumour activity and inhibition of some enzymes participating in carcinogenesis. Because of that

and also due to their plant origin, they have become the most popular chemopreventive compounds. Flavonoids are widely considered as safe, nontoxic natural compounds, which possess significant properties and the consumption and use of dietary supplements containing flavonoids dramatically increased over the past years [60].

Their most discussed characteristic is their capability to scavenge reactive oxygen species such as HO•, NO•, H_2O_2, RO• and superoxide anions. These reactive intermediates, contribute to the stress-related diseases such as cardiovascular diseases, neurodegeneration, and carcinogenesis. Flavonoids also act as antioxidants by directly chelating metal ions, but they are also involved in the highly aggressive HO• conversion [61].

Inhibition of drug metabolizing enzymes CYPs, which are necessary for some carcinogen activations, is a chemopreventive property of various flavonoids. These processes have been extensively studied because of their ability to block the initiation stage of carcinogenesis. Furthermore, flavonoids also inhibit many enzymes that are targets in anticancer treatment, e.g. topoisomerase I and II, Cox I and II and estrogen 2- and 4-hydroxylases [58].

Despite their natural origin and all beneficial properties, the adverse effect may occur as well. It has been suggested that flavonoids may act as mutagens, pro-oxidants and inducers of enzymes participating on procarcinogen activation. Flavonoids apigenin and naringenin, upon oxidation by H_2O_2, formed phenoxyl radicals which catalysed glutathione co-oxidation, resulting in *reactive oxygen species* (ROS) formation [62]. As another example, flavonols containing catechol or pyrogallol B rings may auto-oxidize in the presence of metal ions to produce ROS, which then accelerate low-density lipoprotein oxidation [63]. Additionally, peroxidase-mediated oxidation of catechol B ring containing flavonoids results in the formation of semiquinone and quinone-type metabolites that may act as reactive electrophiles and form adducts with several macromolecules [64]. One of the most abundant flavonoid, quercetin, has been shown to covalently bind to human serum albumin in its reactive oxidized form [65].

1.4.3. Selected flavonoid compounds

Some individual flavonoids selected for the experimental part of this thesis are discussed bellow.

Myricetin

Myricetin is a naturally occurring flavonol that is found in many vegetative foods such as walnuts, berries, fruits, vegetables, herbs, grapes and red wine. Myricetin possesses antioxidant properties, and the consumption of myricetin correlates with a lower risk of some types of cancer [66]. Estrogenic activity of myricetin might be considered as a potential factor in the association of red wine intake and breast tumours, particularly in postmenopausal women [67]. It has also one of the flavonoid exerting inhibitory effects on sulfotransferase activity [28].

Dihydromyricetin

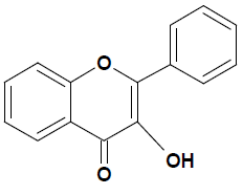
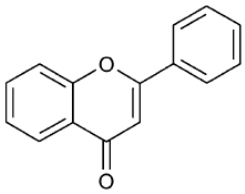
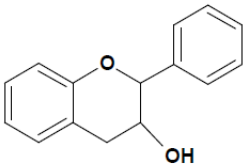
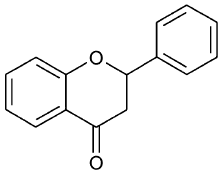
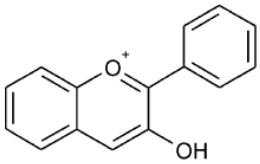
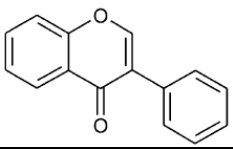
Dihydromyricetin was isolated from the tender stem and leaves of the *Ampelopsis grossedentata* species (dihydromyricetin is also named as Ampelopsin). Most people, however, know another source of this flavonoid compound - *Hovenia dulcis* (known as "Japanese raisin tree") [68,69]. In East Asia, *H. dulcis* has long been used in traditional herbal medicine for the treatment of liver diseases and detoxification after alcoholic poisoning. Current studies put much effort to further investigate the properties of *H. dulcis* based on its hepatoprotective effects [70]. Furthermore, *H. dulcis* is intensively studied for its premier anti-hangover effects. It is believed, that dihydromyricetin is a flavonoid compound, which helps to relieve hangover partly by promoting ethanol elimination *via* enhancement of alcohol dehydrogenase and acetaldehyde dehydrogenase activity [71].

β - Naphthoflavone

β-naphthoflavone (BNF) is a synthetic flavonoid with the structure similar to flavones (with one extra benzene ring). It is generally believed that BNF is able to alter tumour initiation through induction of enzymes involved in the metabolism of carcinogens, especially CYP1A1 and CYP1A2 thus serves as a chemopreventive agent. Indeed, it has been shown that BNF as an Ah-receptor agonist modulated aflatoxin B1 metabolism and inhibits the mutagenic and hepatocarcinogenic activity of aflatoxin B1 metabolite [72,73].

However, some compounds undergo metabolic activation by phase I and phase II enzymes. Thus such an induction leads to an adverse effect - potentiation of bioactivation. It has been shown that sulfation activities towards 4-OH-propranolol (a sympatholytic non-selective beta blocker) were increased in Hep G2 cells by pretreatment with BNF and cell toxicities of 4-OH-propranolol were attenuated in BNF-pretreated Hep G2 cells when compared to control cells [74]. Examples, chemical structure and main dietary sources of other flavonoids are briefly presented in Tab. 2.

Table 2: Chemical structures, examples, and major food sources of 6 major flavonoid subgroups. *Adapted from* [75,76].

Flavonoid group	***Chemical structure***	***Examples***	***Food sources***
Flavonols		quercetin kaempferol myricetin galangin rutin	onion apples curly kale broccoli tea red wine
Flavones		apigenin luteolin baicalein tangeretin	parsley celery sweet, red peppers
Flavanols (Catechines)		catechin epicatechin epigallocatechin	chocolate green tea beans cherry red wine
Flavanones		hesperidin naringenin	citrus fruits
Anthocyanins		cyanidinpelargonidin	berries grapes cherries
Isoflavones		biochanin A genistein diadzein	soy legumes

1.5. Antibodies

Inducible enzymes participating in metabolic activation can be modulated by phytochemicals (e.g. flavonoids), thus it is necessary to consider and assess these interactions. One of the approaches, how to determine the changes in gene expression of drug metabolizing enzymes, is immunoblotting. In this Thesis, antibodies against peptide antigens representing rat sulfotransferases have been prepared in order to determine the changes at the protein level.

Antibodies (members of the immunoglobulin superfamily, also called immunoglobulins) are soluble glycoproteins that are capable of recognizing antigens. Such recognition is the hallmark of the adaptive immune response of vertebrates. Immunoglobulins are expressed either as membrane-bound receptors on the surface of lymphocytes B (B cells), or as soluble molecules (secreted from plasma cells) present in serum and tissue fluids. Specific interaction between an antigen and antibody is mediated by multiple non-covalent bonds. These attractive forces that can together generate a high-affinity interaction including:

- hydrophobic forces between hydrophobic groups,
- attractive forces between induced dipoles (van der Waals / London forces),
- hydrogen bridge formation,
- Coulomb forces between opposed charges (e.g. COO^- and NH_3^+).

Since the antibodies act as the adapter molecules for different immune effector systems, several cases may subsequently occur: (i) immune complex can activate the complement classical pathway, (ii) antibodies bound on pathogens cause opsonization (they mark them) for phagocytosis, (iii) antibodies bound to cells can promote their recognition and killing by NK cells, (iv) and finally the antibody can sensitize cells and induce its apoptosis [77].

1.5.1. General structure

The general structure of an antibody (similar to letter Y) consists of 4 polypeptides - two light chains (L) and two heavy chains (H), linked by a disulfide bond. The heavy and light chains are aligned so that the N-terminal end of a single heavy and a single light chain form an epitope-binding site, the so called Fab fragment. The C terminal ends, on the other hand, form so the called Fc fragment. Furthermore, each chain can be subdivided into a

homologous region - variable and constant domains (see Fig.5). The molecular weight of antibodies is usually 150 to 190 kDa, depending on its class and subclass [78]. The most abundant immunoglobulins belong to type G (IgG), next IgA, IgM, IgD, and IgE.

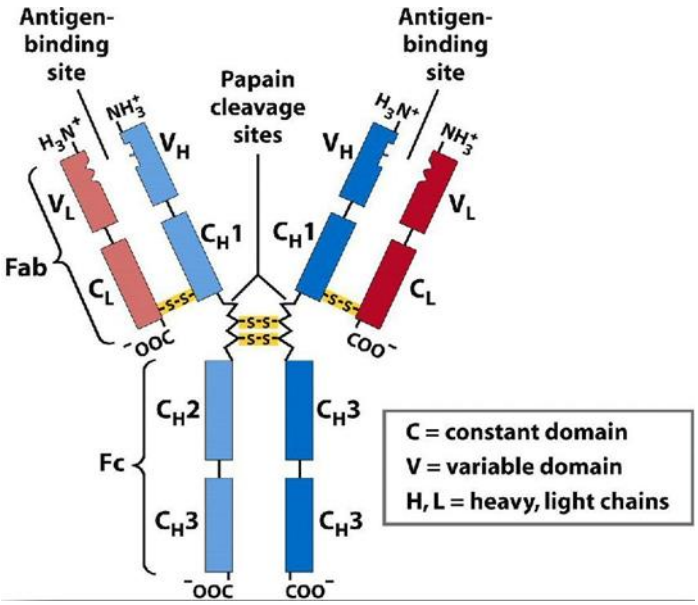


Figure 5: General structure of the immunoglobulin type G (IgG). *Two identical "heavy" chains are composed of four domains (blue). Two identical "light" chains having two domains are assembled by disulfide bonds (red). Cleavage with papain separates the Fab and Fc fragments in the hinge region. Adapted from* [79].

1.5.2. Immunoglobulin classes

The immunoglobulins may be divided into five different classes, based on the differences in the amino acid sequences in the constant region of the heavy chain (type of heavy chain is marked by letters form Greek alphabet) - IgG (γ), IgA (α), IgM (μ), IgD (δ), and IgE (ε).

1. **IgG class**

IgG is the most versatile monomeric immunoglobulin, responsible also for most of the classical adaptive immune response. It accounts for 70-75% of the whole immunoglobulin pool. There are 4 subclasses IgG1-IgG4, which are present in the blood serum in approximate proportions of 66%, 23%, 7%, and 4%. They mutually differ in their

functions - opsonisation, complement activation, adaptive immune response, and toxin neutralisation [77]. Molecules of IgG have the above described general structure, which is often used as a model immunoglobulin.

2. IgA class

Immunoglobulins of class A are found mainly on mucous surfaces and in mucous secretions and they account for approximately 15-20% of the serum immunoglobulin pool [77]. Their main function is to prevent infection agents, mainly bacteria and viruses from breaching the physical barrier of tissues. Secreted IgA protein structure (SIgA, serum Ig) is similar to IgG one, except that they form dimers, connected by the so called joining chain (J-chain).

3. IgM class

IgM are mostly of pentameric structures, occurring in mammal serum, representing 10% of all antibodies. Individual monomers are covalently linked together with disulfide bonds and together they provide ten binding sites, possessing significant avidity for antigens. However, IgM occur also as monomeric immunoglobulins, as an integrated part of the B cell membranes (B cell receptor; BCR). In this form, they serve as starters of the specific immune response. In addition, pentameric IgM form immune complexes with antigens that subsequently activate the classical complement pathway.

4. IgD class

Isotypes D of immunoglobulins occur in both forms - as secreted antibodies (approximately 1% of the Ig pool) and also in the plasma membrane of mature B cells, as receptors. Regarding its function, IgD have remained a mysterious antibody class since their discovery in 1964 [80]. Recently, it has been found that circulating IgD bind to basophils. These complexes of IgD-basophil are then capable of inducing antimicrobial, opsonizing, inflammatory, and B cell stimulating factors such as interleukins and cathelicidins. Thus it seems that IgD lead the surveillance system at the interface between immunity and inflammation [81].

5. **IgE class**

Immunoglobulins of isotype E are also monomeric and are present in even smaller concentration than IgD - less than 0.05 μg/ml. Their main function includes immunity to parasitic worms and to certain protozoan parasites. Furthermore, IgE also play an essential role in hypersensitivity type I, since they mediate the interaction between antigen and mast cells/basophils through specific Igε receptors. Activated mast cells then release mediators from their granules, e.g. histamine, which triggers in this case the process of vasodilatation [77].

1.5.3. Antibody preparation and usage

Not only human health can profit from antibody abilities. Several assays (immunoassays) measure the concentration or detect the presence of a macromolecule in a solution through the use of antibodies. Moreover, most immunoassays involve chemically linking antibodies or antigens to some kind of a detectable label - radioisotopes (radioimmunoassay), enzymes (enzyme-linked immunosorbent assay ELISA, immunoblotting techniques), dyes (immunofluorescence) etc.

There are two types of antibodies, based on antibody origin - monoclonal and polyclonal. Polyclonal antibodies recognize several independent epitopes on an antigen and each of them is produced by a different clone of the plasma cell. They are usually heterogeneous at all levels (specificity, classes, and subclasses, titre, and affinity) and are used in immunoassays mentioned above. The main source is usually the serum of a previously immunized animal. Traditionally, commercially available polyclonal antibodies are produced from the sera of mice, rats, rabbits, sheep, goats, and horses, or from egg yolks of immunized hens. However, the stimulation of an antibody production may vary by antigen used. Commonly used antigens include natural, artificial, and synthetic antigens or coupling haptens/peptides representing some protein with a carrier molecule [82]. Monoclonal antibodies on the other hand are highly specific glycoproteins produced from hybridoma cells. Hybridoma cells are typically made by fusing myeloma cells (immortal cell line) with the spleen cells from a mouse that has been immunized with the desired antigen. Each clone is grown from the single parent cell separately, thus only one clone can produce only one antibody type. The main disadvantage is that the whole procedure is very

demanding and expensive. The main advantage, the high specificity, is required. for example, in therapeutic applications [83].

Recently, antibodies from egg yolks of immunized hens have become suitable alternative to conventional antibodies from the blood of experimental animals. The characteristics and advantages of this approach are briefly described in the next chapter.

1.5.4. Chicken antibodies from egg yolks

The use of eggs from immunized chicken as an inexpensive and rich source of polyclonal antibodies has become more significant for biochemistry research. However, it is still less common than mammal sources because of the lack of quality purification protocols. Like humans, chicken protect their offspring by transferring maternal antibodies from serum to the egg yolk, from which they can be then purified [84]. Birds have only 3 classes of immunoglobulins: IgG, IgA, and IgM. During egg fertilization, IgG start to concentrate in yolk, while IgM and IgA are secreted into egg white [85]. However, IgG of mammals and birds are not identical, therefore a new nomenclature IgY (the "Y" in IgY comes from "yolk") has been introduced. The IgY (Fig.6) contains two heavy chains and two light chains and has a molecular mass of 180 kDa (in comparison with 159 kDa). IgY possesses larger molecular weight of heavy chain (68 kDa) as compared to 50 kDa in mammals. Furthermore, heavy chain of IgY does not have a hinge region and possesses four constant domains in addition to the variable one [86]. IgY have lower isoelectric point and are incapable of activation of mammalian complement system [87].

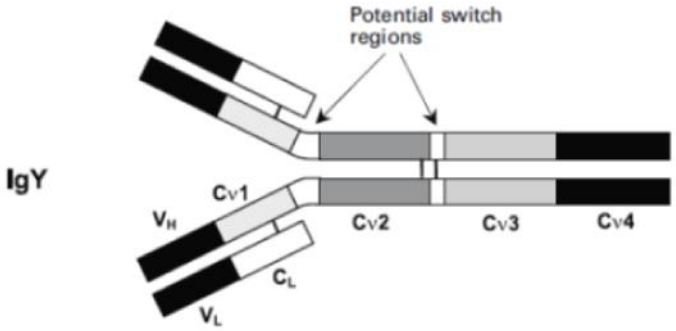


Figure 6: Structure of IgY. *V_H = variable heavy chain, V_L = variable light chain, C_L = constant light chain, C_υ = epsilon chain. Adapted from* [88].

The use of chicken antibodies from egg yolks has a number of advantages over the mammal ones generated from sera (summarized in Tab.3):

→ **non-invasiveness and cost-effective care**

Non-invasive collection of eggs is incomparable with obtaining serum, which can be stressful and painful. Furthermore, chicken care is cheaper and the sustained high titre of antigen reduces the need for injection repetition.

→ **enhanced immunogenicity due to the phylogenetic distance**

Due to the phylogenetic distance between mammals and birds, the complex formation is usually more successful than in other mammals [84]. They will bind to more epitopes than a corresponding mammalian antibody, which can in the end amplify the output signal. In addition, IgY antibodies tend to recognize the same protein in a number of mammalian species [85].

→ **higher yield**

Compared to IgG obtained by conventional immunization methods, almost 8x higher amount of immunoglobulins can be harvested each month [85]. Although the immunoglobulin concentration in yolk (10–25 mg/ml) is lower than in mammalian serum (e.g. for rabbit – 35 mg IgG/ml), the total productivity of antibodies speaks in favour of hens, since they lay about 20 eggs per month [89].

→ **fast and simple process of antibody isolation by simple precipitation techniques**

Egg yolk proteins are generally distributed in two particular parts: the granules and the plasma. IgY, as plasmatic proteins, are isolated and purified from the complex egg yolk matrix, requiring specific methods. There are several approaches for IgY isolation, as already reviewed [90]. Generally, the purification of IgY from separated yolks usually proceeds in two stages: (i) the water-soluble fraction containing IgY is separated from the lipidic fraction composed mainly of lipids and lipoproteins, and (ii) IgY are isolated from the other soluble proteins [91]. Lipophilic yolk components are removed by conventional centrifugation or filtration, while precipitation step is performed using various solvents (polyethylene glycol, dextran sulfate, alginate, sodium chloride etc.).

→ **IgY does not bind to the mammalian or bacterial Fc receptor, it does not activate the complement system or it interacts with the rheumatoid factor**

Rheumatoid factor (RF) is an autoantibody, which can occur in patient serum during some diseases e.g. rheumatoid arthritis, but it is also present in red blood cells of healthy

individuals. RF is thus one of the major sources of interference in many immunoassays. When "sandwich" ELISA is performed, primary and secondary labelled antibody is used to detect the antigen. When RF factor with an affinity to the same antibodies as the detected antigen is present, false positives results occur [92].

Table 3: Comparison between mammalian IgG and chicken IgY. *Adapted from* [86].

Animal	Rabbit (IgG)	Chicken (IgY)
Source of antibody	blood serum	egg yolk
Kind of antibody	polyclonal	polyclonal
Antibody sampling	bleeding	collecting eggs
Antibody amount	200 mg / serum sample	100-150 mg /egg
Quantity of antibody per year	1400 mg	40 000 mg
Amount of specific antibody	5%	2-10%
Protein A/G binding	Yes	No
Interaction with mammalian IgG	Yes	No
Interaction with rheumatoid factor	Yes	No
Activation of mammalian complement	Yes	No

2. AIMS OF THE STUDY

This thesis is focused on phase II metabolism enzymes, sulfotransferases, which are involved in biotransformation of carcinogens. Administration of chemopreventive compounds may induce them and thus increase the activation of carcinogens. To study the effects of chemopreventive flavonoids (α-naphthoflavone, β-naphthoflavone, myricetin, dihydromyricetin) *in vivo* on the induction of sulfotransferases, following tasks should be accomplished:

- To isolate and purify specific chicken antibodies recognizing rat cytosolic sulfotransferases in order to use them for immunodetection by Western blot technique,
- To establish and optimize sulfotransferase activity assay using the HPLC method,
- To determine the effect of flavonoid compounds (myricetin, dihydromyricetin, α-naphthoflavone and β-naphthoflavone) and/or carcinogens (PhIP, benzo[*a*]pyrene) on the protein expression of sulfotransferases, using isolated anti-rat antibodies, and
- To determine the activity of the sulfotransferases in cytosol samples of treated rat models, using the HPLC method.

3. MATERIALS AND METHODS

3.1. Used materials

Cypex

Human recombinant SULT1A1*1 expressed in Escherichia coli (stored at -80°C)

Fluka, Schwitzerland

p-nitrophenyl phosphate (pNPP), tris(hydroxymethyl)aminomethane (Tris), β-mercaptoethanol

Lach-Ner, Czech Republic

sodium chloride (NaCl), monosodium phosphate (NaH_2PO_4), hydrochloric acid (HCl), sodium carbonate (Na_2CO_3), sodium bicarbonate ($NaHCO_3$), disodium phosphate (Na_2HPO_4), magnesium chloride ($MgCl_2$), ethylenediaminetetraacetic acid (EDTA), potassium phosphate (KH_2PO_4), ammonium persulfate (APS), acetic acid (CH_3COOH), methanol (MeOH), glycine, potassium hydroxide (KOH), phosphoric acid (H_3PO_4)

Merck, Germany

Acetonitrile (CH_3CN)

Millipore Corp., USA

Immobilion - NC transfer nitrocellulose membrane

Penta, Czech Republic

sodium hydroxide (NaOH), sodium azide (NaN_3), ethanol (CH_3CH_2OH)

PML a. s., Czech Republic

nonfat powdered milk Laktino

R&D Systems

3'-phosphoadenosine-5'-phosphate in 10 mM sodium borate (PAP)

Serva, Germany

Tween-20, sodium dodecyl sulfate (SDS), Tris/HCl, acrylamide, bis-acrylamide (BIS), N,N,N',N'-tetramethylethylendiamine (TEMED), Coomassie brilliant blue R-250 (CBB), Triton X-100, adenosine-diphosphate (ADP), L-cysteine·HCl

Sigma-Aldrich, USA

dimethylsulfoxide (DMSO), diethylamine (DEA), guanidin/HCl, 5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium tablets (BCIP/NBT), alkaline phosphatase-conjugated rabbit anti-chicken IgG, p-nitrophenol (pNP), p-nitrophenyl sulfate (pNPS), triethylamine, 3′-phosphoadenosine - 5′-phosphosulfate lithium salt hydrate (PAPS), tetrabutylammoniumbisulfate (TBAS)

Thermo Fisher Scientific, USA

ImjectMaleimide-Activated mcKLH, Microplate F16 MaxiSorp NUNC-IMMUNO MODULE, Sulfo Link® Coupling Resin, Microplate BCA Protein Assay Kit – Reducing Agent Compatible, PageRuler Unstained Broad Range Protein Ladder, PageRuler Prestained Protein Ladder

Whatman, USA

Whatman paper, No.3

3.2. Used devices

Analytical balance

Discovery, Ohaus, USA

Automatic micropipettes

Multi pippet Proline, Biohit, Finland

Balance

Kern EW 600-2M, Kern&Sohn, Germany

Centrifuges

Centrifuge 5418, Eppendorf, Germany

K70, Janetzki, Germany

Concentrator

CentriVap Concentrator, Labconco, USA

Cooker

Cooker ETA, Czech Republic

Dispenser repeater

Dispenser Repeater®, Eppendorf, Germany

Dryer

HydroFlex, Tecan, Switzerland

Electrophoresis equipment

Mini-PROTEAN Tetra System, BioRad, UK

HPLC systems

I.

Pump: Dionex pump P580

ASI-100 Automated Sample Injector

UV/VIS Detector UVD 170S/340S (USA)

Degasys DG-1210, Dionex

Thermobox: COLUMN OVEN LCO 101

Column: Macherey-Nagel, Nukleosil 100-5 C18 HD; CC 4 x 250 mm (Germany)

Programm: Chromeleon™ 6.11 build 490

II.

Agilent Technologies 1200, USA

Pump: Quaternary pump 1200

1200 Standard Autosampler ALS G1329A

1200 Vacuum degasser G1322A

Column oven LCO 102, ECOM s.r.o.

Guard Cartridges Chromolith® 10-4.6mm RP-18e, Merck

Incubator

IR 1500 Automatic CO_2 Incubator, Flow laboratories, UK

Thermomixer compact Eppendorf (Germany)

Magnetic stirrer

KMO 2 basic IKA®- WERKE, IKA, Germany

Microliter syringe

Microliter syringe 702RN, Hamilton, Switzerland

Microplate reader

Microplate absorbance reader Sunrise, TECAN, Switzerland

pH meter

HI 2211, HANNA Instruments, USA

Pippetes

Nichipet EX, Nichiryo America, Inc., Japan

Power supply

EPS 301 Power supply, Amersham Biosciences, USA

Protein detection system

SNAP i.d®, Millipore corp., USA

Shaker

Shaker Vyvojovedilny, Czechoslovakia

Sonicator

Elmasonic E 30 H, P-Lab, Czech Republic

Spectrophotometr

SpectroMOM 195 D, MOM, Hungary

Vortex

MS 1 minishaker, IKA, Germany

Water purification system

Milli-Q® Simplicity 185, Millipore corp. USA

Western blot equipment

TransBlot® Turbo™ Transfer System, BioRad, UK

3.3. Methods

3.3.1. Preparation of anti-peptide antibodies

3.3.1.1. *Design of peptide antigen*

Instead of using the whole proteins (enzymes) as antigens, whose preparation and purification can be very expensive and demanding, peptide antigens were designed to serve as complex antigen biomolecules [93]. In order to maximize the probability that the antibodies would recognize the native protein in the target assay, antigen design was carefully planned according to the following criteria: antigenicity, exposition (surface-orientated), hydrofility, absence of secondary structure, unique sequence of protein, and peptide length.

With the help of specialized online modelling tools for the prediction of peptide properties, 7 peptides (9 - 15 amino acids) representing rat sulfotransferases were designed and provided by Prof. RNDr. Petr Hodek, CSc. (peptides were synthetized by VIDIA, Czech Republic).

Peptide sequences are shown in Table 4.

Table 4: Amino acid sequences of designed peptide antigens

Enzyme	Amino acid sequence
rSULT1A1	TDCDFKFR**C**
rSULT1B1	LNDGNVEK**C**
rSULT1C1	TLPSSIMD**-C**
rSULT1C2	KMGGTSLNF**C**
rSULT1E1	**C**PVKFRAEL
rSULT2A1	**C-**SNYSLLMKK
rSULT1C1/2	SPFMRKGTVGDWKN**-C**

-C means that cysteine was not originally a part of the sequence, but that it was introduced for further conjugation to the carrier protein

3.3.1.2. *Conjugation of peptides with a protein carrier*

Peptides (haptens) themselves are too small to elicit an immune response. Maleimide activated mcKLH (modified keyhole limpet hemocyanin) serves as a conjugation protein, since it has a significant immunogenicity and ability to bind cysteine (internal cysteine was present in peptide or was synthetized at the end of the sequence). Maleimide groups react with free sulfhydryls, forming stable thioether bonds (Fig. 6). As a result, conjugated hapten effectively elicits an immune response and antibody production.

First, a 10 mg/ml mcKLH water solution was incubated at room temperature for 20 min. Peptides were dissolved in a conjugation buffer (0.1 M sodium phosphate buffer; 0.15 M NaCl; pH 7.2), in the same concentration as mcKLH. Both solutions were mixed in 1:1 ratio (200 µl of peptide + 200 µl of mcKLH) and left for 2 hours at room temperature, while stirring. Each of the final peptide conjugate had a concentration of 5 µg/µl. Samples were aliquoted and stored at -20°C.

Some peptides were more hydrophobic than others, which was manifested as a formation of clots while dissolving, so they needed to be dissolved in dimethylsulfoxid (DMSO) before using PBS. Therefore, the amount of 2 mg of peptide was mixed with 70 µl DMSO, and was sonicated for 5 minutes. After short centrifugation (3 min, RPM 10 000), the supernatant was removed and 100 µl DMSO was added again. Mixtures were sonicated for another 2 minutes until clear solution was observed. Finally, 100 µl of PBS was added into the tube, solution was mixed and further used for conjugation according to above described procedure.

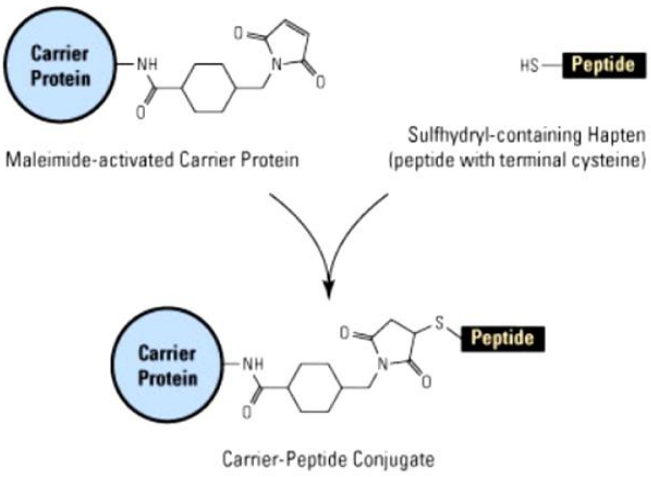


Figure 6: Reaction scheme of mcKLH conjugation with SH-containing peptide.
A*dapted from* [94].

3.3.1.3. *Antibody isolation from chicken egg yolk*

For antibody isolation and purification, two pools of eggs were used. The first pool contained eggs collected before the immunization of hens (control) and the second one contained eggs from the period of 6 weeks after the last dose of antigens. Table 5 shows number of eggs used for isolation.

Table 5: Collected eggs used for IgY isolation

IgY	control	SULT1A1a	SULT1A1b	SULT1B1	SULT1C1
eggs	6	7	5	9	7
IgY	SULT1C2	SULT1C1/2	SULT1E1	SULT2A1a	SULT2A1b
eggs	10	9	6	9	5

a - first immunization batch; b - second immunization batch

There are several methods for chicken antibody isolation, as previously described (Chapter 1.5.4). Here we used the procedure based on freezing-thawing of the diluted yolks followed by sodium chloride precipitation at low pH [91]. Egg yolks were separated from whites, washed with tap water and pooled in a cylinder. The pool was then diluted with tap water in ratio of 1:7 and the pH value was adjusted to 5.0 with 0.5 M HCl. The mixture was then filled into corked funnel and left overnight in a -20°C freezer.

Next day, during the spontaneous thawing, the frozen suspension was filtrated through a filter paper to eliminate lipoid egg yolk fraction. Next, sodium chloride was added as a precipitating agent in a final concentration of 8.76% and pH was adjusted to 4.0. The IgY protein was precipitated for 30 min while stirring and left standing for additional 90 minutes at room temperature. Finally, the precipitate was sedimented at 3000*g* for 20 minutes at 4°C (Janetzki K70). Supernatants were discarded and the pellets were dissolved in about 15 ml of PBS (containing 0.1% sodium azide). IgY solutions were stored in tubes at 4°C. If turbidity occurred, the solution was centrifuged at 13 000*g* for 5 min (Centrifuge 5418, Eppendorf) and placed into a new clean tube. Protein concentration was detected by measuring absorbance of samples at 280 nm (SpectroMOM 195 D) with PBS (containing 0.1% sodium azide) as a reference, according to formula:

$$\mathbf{c = A_{280} \cdot f}$$

where c = concentration of proteins; A_{280} = absorbance at 280 nm; f = 1.094 (empiric factor)

3.3.1.4. *ELISA*

To determine the specificity of isolated antibodies, indirect ELISA (Enzyme-Linked Immunosorbent Assay) was used. This method makes use of enzymes attached to the secondary antibody to allow quantification through the development of colour after an addition of a suitable substrate. Firstly, the solid phase of microtitre plate is coated with an antigen. Secondly, a specific primary antibody is added, followed by incubation with compatible secondary antibody conjugate. Between each step, the plate is washed to remove any proteins or antibodies that are not bound. After the final washing step, the plate is developed by adding a chromogenic substrate to produce a visible signal, which is finally spectrophotometrically measured.

Reagents:

Immobilization buffer: 15 mM Na_2CO_3, 35 mM $NaHCO_3$; pH 9.6

Washing agent (PBS-Tween 20): 13.4 mM NaCl, 1.8 mM Na_2HPO_4, 1 mM NaH_2PO_4, 0.1% (v/v) Tween 20; pH 7.2

Blocking buffer: 2% (w/v) egg white solution in PBS-Tween 20

PBS: 13.4 mM NaCl, 1.8 mM Na_2HPO_4; 1 mM NaH_2PO_4; pH 7.2

Substrate solution: 1 mg/ml p-nitrophenyl phosphate (pNPP), 0.02 M Na_2CO_3, 0.03 M $NaHCO_3$, 1mM $MgCl_2$

All solutions and samples (standards included) were incubated for at least 20 minutes at room temperature before handling.

A microtitre plate (F16 MaxiSorp NUNC-IMMUNO MODULE) was coated with an antigen solution, which was prepared by the dilution of 1 mg/ml antigen to the concentration 4 μg/ml by an immobilization buffer. An antigen solution (100 μl) was added to each well and the plate was incubated O/N at 4°C.

The next day, the unbound antigen was washed away by a washing agent (3x200 μl). Then, 100 μl of blocking solution was added into each well and the plate was incubated at 37°C for 1 hour. After that, the microtitre plate was washed (3x200 μl) and the primary antibody was added at serial dilutions 90, 30, 10, and 3.3 μg/ml. After the plate incubation at 37°C for 2 hours, the unbound antibody was washed away (3x200 μl) and 100 μl of alkaline phosphatase-conjugated rabbit anti-chicken IgG was added into the wells

(dilution 1:1 400). The plate was again incubated at 37°C for 1 hour and then finally washed (3x200 µl). Subsequently, 100 µl of substrate solution with pNPP was added and the reaction was stopped after 10 minutes by adding 100 µl of 3 M NaOH. The absorbance measurement at 405 nm was immediately performed (Microplate absorbance reader Sunrise) and data were evaluated.

3.3.1.5. *Affinity purification of anti-rat SULT1A1 antibody*

Because the reactivity of the anti-peptide antibodies was not specific and they displayed cross-reactivity with other antigens, affinity purification was performed. In this process, we took advantage of the cysteine present at the end of the peptide sequence. Sulfhydryl-containing peptide was immobilized to agarose beads (SulfoLink® Coupling Resin) by means of reaction with iodoacetyl groups, resulting in the thioether bond assembly. Once the peptide was immobilized, the affinity column was filled with stationary phase and incubated with target antibody solution. The fraction of specific antibodies was finally released as an eluent and used for immunoassay.

Reagents:

Coupling buffer: 50 mM Tris/HCl, 5 mM EDTA; pH 8.5

Washing buffer: 1.0 M NaCl

Blocking buffer: 50 mM L-cysteine·HCl, 50 mM Tris, 5 mM EDTA; pH 8.5

Storage buffer: PBS, 0.1% (w/v) NaN_3

Elution buffer: 50 mM diethylamine; pH 11.5

Neutralization buffer: 1 M potassium phosphate, pH 6.7

Procedure for peptide immobilization

Synthetized peptide rSULT1A1 was dissolved in 2 ml of coupling buffer in a final concentration of 0.75 mg/ml. Since the peptide contains hydrophobic amino acids and was not readily soluble, it was first dissolved in 200 µl of DMSO. The required volume was then attained by the addition of buffer. The mixture was incubated for 10 minutes at room temperature. In the meantime, affinity column was filled with 4 ml of suspense SulfoLink® Coupling Resin, giving 2 ml of the gel, and washed with 8 ml of coupling buffer. The gel was mixed with 2 ml of the peptide-solution on end-over-end mixer for 15 minutes

followed by 30 minutes incubation upright at room temperature. To remove unbound peptide, the column was then washed by 8 ml of coupling buffer with 20% DMSO, followed by 8 ml of coupling buffer. Residual binding capacity of the gel was blocked by 2 ml of blocking buffer and the solution was mixed 15 minutes on end-over-end mixer, followed by 30 minutes incubation upright at room temperature. In the next step, the flow through was discarded. The column was then washed in order to release weakly bound antigen by (i) 16 ml of 1 M NaCl; (ii) 6 ml of diethylamine, pH 11.5; (iii) 6 ml of 100 mM KH_2PO_4; pH 7.2, and (iv) 6 ml of PBS.

Affinity purification of antibodies

Solution of anti-rat SULT1A1 antibody (20 ml) was incubated with immobilized peptide on SulfoLink® Coupling Resin on end-over-end mixer, O/N at 4°C. Next day, the unbounded IgY fraction was collected for ELISA assay. The column was repeatedly washed with PBS until the absorbance at 280 nm of the flow through reached 0.04. Fractions of weakly bound antibodies were eluted with 13 ml of 1M NaCl in PBS, and collected into Eppendorf tubes. Those with absorbance values < 0.2 were pooled. After the washing step with 15 ml of PBS, specific antibodies were eluted with elution buffer and 1 ml-fractions were collected into Eppendorf tubes containing neutralization buffer (200 μl). Fractions with the highest amount of protein were pooled and dialyzed against 5 l of storage buffer O/N at 4°C. The column was finally washed with storage buffer and stored at 4°C.

3.3.2. Determination of protein concentration

Protein concentrations of cytosol samples were determined using Thermo Scientific™ Pierce™ BCA Protein Kit, based on method by Wiechelman et al. [95], and BSA as protein standard.

It has been shown, that cysteine, cystine, tryptophan, tyrosine, and the peptide bond are capable of reducing a copper cation Cu^{2+} to a cuprous cation Cu^{+}. In alkaline environment, bicinchoninic acid (BCA) forms with Cu^{+} blue-violet complexes. This water-soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentration. As the final colour continues to develop, immediate absorbance detection is necessary.

Reagents:

Reagent A: 2% (w/v) $Na_2CO_3.H_2O$, 0.95% (w/v) $NaHCO_3$, 0.16% (w/v) sodium tartarate, 0.4% (w/v) NaOH

Reagent B: 4% (w/v) $CuSO_4.5H_2O$

BCA working reagent: 50 parts of reagent A + 1 part of reagent B + BCA in a final concentration of 1% (w/v)

Protein concentration was determined in rat cytosol. Cytosolic fractions prepared from rat liver tissue samples were kindly provided by Mgr. Eliška Brabencová [96]. Cytosolic fractions prepared from rat colon tissue samples were obtained from Bc. Petra Fousová (unpublished). Samples were stored aliquated at -80°C until used. Selected chemopreventive compounds and carcinogens were used for a one dose-administration or sequential administration. The administration schemes are listed in *Appendix 1*.

Cytosolic samples were prepared with a different dilution in duplicates and kept in sterile Eppendorf microtubes. Standards for the calibration curve were prepared by diluting the Albumin Standard Ampule (2 mg/ml) with distilled water to concentrations 1.5; 1.0; 0.75; 0.5; 0.25 and 0.125 mg/ml. To reduce the error probability, the standards were measured in duplicates and samples in triplicates, respectively.

Samples were pipetted into 96-well plate in total volume of 9 μl each (blank sample contained distilled water). After that, 260 μl of freshly prepared BCA working reagent was added. Covered plate was incubated at 37°C for 30 minutes (IR 1500 Automatic CO_2 incubator). Finally, the absorbance was measured on a plate reader at 562 nm (Sunrise Absorbance Reader). Data was evaluated by the Kim32 program.

3.3.3. SDS-polyacrylamide gel electrophoresis

Electrophoresis is a widely used separation technique based on molecular weight and charge of macromolecules. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) uses the ability of anionic detergent sodium dodecyl sulfate (SDS) to confer a negative charge to a polypeptide chain, thus allowing proteins to separate according to their molecular weight, as they pass through the gel.

Gel solutions:

Resolving gel: water
1.5 M Tris/HCl, pH 8.8
30% (w/v) acrylamide mix
10% (w/v) SDS
Tetramethylethylenediamine (TEMED)
10% ammonium persulfate (APS)

Stacking gel: water
1.5 M Tris/HCl, pH 6.8
30% (w/v) acrylamide mix
10% (w/v) SDS
Tetramethylethylenediamine (TEMED)
10% ammonium persulfate (APS)

4X Sample buffer: 0.25 M Tris/HCl, 8% (w/v) SDS, 20% (v/v) 2-mercapthoethanol, 40% (v/v) glycerol, 0.003% (w/v) bromphenol blue; pH 6.8

Electrode buffer: 0.195 M glycine, 0.025 M Tris/HCl, 0.1% (w/v) SDS; pH 8.3

Staining bath: 0.25% Coomassie brilliant blue R-250, 46% (v/v) ethanol, 9.2% (v/v) acetic acid

Distaining bath: 25% (v/v) ethanol, 10% (v/v) acetic acid

For 2 gels:

Resolving gel 10%: 4 ml of distilled water, 3.3 ml 30% acrylamide mix, 2.5 ml 1.5 M Tris/HCl, 100 µl 10% SDS, 100 µl 10% APS, 2 µl TEMED

Stacking gel 3%: 2.3 ml of distilled water, 300 µl 30% acrylamide mix, 380 µl 1.5 M Tris/HCl, 30 µl 10% SDS, 30 µl 10% APS, 3 µl TEMED

First, the multi-casting chamber with particular glasses was constructed and gels were prepared. The 10% resolving gel was applied between the glasses (10x8.2; 10x7.4; 1 mm thickness), immediately overlaid with distilled water and allowed to polymerize for 30 minutes at room temperature. Next, water was removed and the area above the gel was dried with filter paper. The 3% stacking gel was poured on top of the resolving gel, the

comb (10 wells) was placed and again, the gel was allowed to polymerize for 15 minutes at room temperature. After the polymerization, the glass-sandwiches were removed from the casting chamber and put into electrophoresis racks, while combs were carefully displaced. Racks were filled with electrode buffer.

Secondly, cytosolic samples were diluted to the final protein concentration 2 mg/ml or 1 mg/ml, respectively, and then were diluted at a ratio 3:1 with the 4X sample buffer. All samples were boiled in 100°C water bath for 5 minutes. The PageRuler Unstained Broad Range Protein Ladder/PageRuler Prestained Broad Range Protein Ladder (5 μl; Thermo Scientific) and samples (20 μl = 30/15 μg) were then loaded into the gel wells by a Hamilton syringe.

The electrophoresis chamber was filled with an electrode buffer and connected to power supply. Electrophoresis ran at constant voltage 200 V until the blue forehead reached the bottom of the gel.

The gels were removed and separated from stacking parts. One was put into a staining bath for 60 minutes and subsequently distained O/N in distaining bath. The second gel was used for Western blotting without staining, as follows.

3.3.4. Western blot

Western blotting is an immunoassay technique for the detection of specific proteins by transferring those from the gel to the blot membrane. After incubation with specific primary antibody, the protein on membrane is detected by a secondary antibody conjugate with the enzyme, which allows visualising target proteins on the membrane.

Typically, the method for transferring the proteins uses an electric current to pull proteins from the gel into the polyvinylidene fluoride or nitrocellulose membrane.

Reagents:

Transfer buffer: 0.025 M Tris, 0.192 M glycine; pH 8.3

PBS Triton-X 100: 0.134 M NaCl; 1.8 ml $Na_2HPO_4.10H_2O$; 1 mM NaH_2PO_4, pH 7.2; 0.3% (w/v) Triton X-100

Blocking solution: 5% fat free milk (PML a.s.)

Following the electrophoresis, the gel was incubated with the transfer buffer (containing 10% methanol) for 20 minutes at room temperature. In the meantime, the nitrocellulose membrane was cut according to gel dimensions, keeping the shape of the gel. Subsequently, the nitrocellulose membrane was wetted in an electrode buffer, then washed in distilled water for 2 minutes and kept in transfer buffer for at least 5 minutes.

An anode electrode plate of the TransBlot® Turbo™ Transfer System was covered with three sheets of Whatman papers No.3, soaked in transfer buffer. The membrane was placed on the top of sheets and then covered with gel. The whole assembly was covered again with 3 sheets of Whatman papers, soaked in transfer buffer. The "sandwich" was neatly compressed and the cathode plate was placed on the top of it. The completed cassette was put into Transfer System and ran for 7 minutes at constant voltage 25 V. When the process of transfer was complete, the membrane was blocked in 5% fat free milk for 1 hour at 4°C in order to prevent non-specific interactions between the membrane and the primary antibody.

After blocking, the membrane was incubated with tested chicken primary antibody at the concentration 30 μg/ml or affinity purified anti-rat SULT1A1 (concentration 3 μg/ml) in blocking solution for 2 hours at room temperature, while shaking. After incubation, the membrane was washed in blocking solution and PBS Triton-X 100. The incubation with anti-chicken secondary antibody was conducted with SNAP i.d®Protein Detection System as follows. Firstly, the membrane was put on MilliQ-wet chambers (protein site down), covered by MilliQ-wet "spacers", closed and placed into a SNAP device, connected to a vacuum pump. Secondly, the membrane was three times washed with PBS Triton-X 100 (3x15 ml) and then incubated with secondary antibody solution (dilution 1:1429) for 10 minutes. Finally, the membrane was again washed three times by PBS Triton-X 100 buffer and placed into distilled water. The visualisation of the specific developed protein bands was carried out with a BCIP/NTB tablet containing 10 mg substrate for alkaline phosphatase. The membrane was incubated in the substrate solution, until bands were shown, then placed into distilled water and dried between filter papers.

3.3.5. Sulfotransferase activity assay

3.3.5.1. *Analysis of 3'-phosphoadenosine-5'-phosphate using High performance liquid chromatography (HPLC)*

Retention times and properties of nucleotide 3'-phosphoadenosine-5'-phosphosulfate (coenzyme; PAPS) and 3'-phosphoadenosine-5'-phosphate (PAP) as a potential marker of sulfotransferase activity were analysed. Subsequently, a test enzyme assay was performed to access the changes in PAP concentration and identify the position of resulting metabolite of the sulfoconjugation reaction, p-nitrophenyl sulfate.

Nucleotides and metabolites were separated and analysed on an HPLC system (Agilent Technologies 1200, USA) with gradient elution of mobile phase A and B:

mobile phase **A**: 70 mM K_2HPO_4, 6 mM tetrabutylammonium bisulfate; pH 6.1

mobile phase **B**: 50% methanol (v/v)

Separation of compounds was performed on two consecutive guard cartridges (Chromolith® 10-4.6mm RP-18e, Merck), with a 40 sec linear gradient of mobile phase B in mobile phase A, with iniciation of the gradient 2 min after sample injection. The flow rate of 2 ml/min was maintained during analysis.

Standards of nucleotides (PAP and PAPS) were prepared by dissolving in sterile water in different concentrations. A 100 μL of solution was put into vial for analysis and/or stored in -20°C until used. Standards of nucleotides were analysed with an injection volume of 20 μl.

The test assay mixture (100 μl) for sulfotransferase activity determination contained:

- 2 μg recombinant human SULT1A1*1 (c= 0.08 mg/ml; Cypex)
- 50 μM PAPS
- 20 μM p-nitrophenol
- TRIS buffer (0.05 M Tris/HCl, pH 7.5 ; 15 mM $MgCl_2$)

Control reaction contained an assay buffer instead of coenzyme. All reagents of the assay mixture were mixed in volumes to achive the above described concentration. The volume of 100 μl was completed by TRIS buffer. Immediately after reaction iniciation by PAPS,

mixtures were gently vortexed and incubated 10/25 minutes at 37°C in open microtubes while shaking (450 RPM; Thermomixer Compact, Eppendorf). The reaction was stopped with 100 μl ice-cold acetonitrile. The assay mixture was, after the addition of acetonitrile, vortexed and centrifuged at 13 000 RPM for 6.5 min. A 100 μl of supernatant was analysed according to above described conditions.

3.3.5.2. *Determination of p-nitrophenyl sulfate as a marker product using HPLC*

The HPLC method for the determination of the SULT activity was conducted according to Cypex [97] with modifications. Sulfate conjugate, p-nitrophenyl sulfate, was separated and analysed using a reversed phase HPLC system [pump Dionex P580, ASI-100 Automated Sample Injector, UV/VIS Detector UVD 170S/340S]. The mobile phase consisted of 5% (v/v) triethylamine (pH 3.0, adjusted with H_3PO_4) and acetonitrile in ratio 66:34 (v/v), it was prepared fresh and degassed by sonication before use. Separation was conducted using Nukleosil 100-5, C18 HD, CC 4 x 250 mm column (Macherey-Nagel) with the flow rate of 1 ml/min in total time 10 minutes per one analysis. The metabolite, p-nitrophenyl sulfate, was detected in the UV region at 300 nm.

The quantification of metabolites was conducted using the values of the area under the curve (AUC), since they are directly proportional to the concentration of the metabolite. Solutions of external standard p-nitrophenyl sulfate were prepared by serial dilution and subsequently analysed, as above described. The amount of metabolite was calculated according to calibration curve. SULT activity was measured as the formation of p-nitrophenyl sulfate per minute, per mg of protein.

3.3.5.3. *Optimization of sulfotransferase activity assay based on p-nitrophenyl sulfate determination*

The assay had to be optimised for several conditions. In this work, several criteria were tested: retention times, incubation time, protein content, sample concentration, solvent, and injection volume. All optimization incubations were conducted with a final protein content of 2 μg of human SULT1A1*1 or 200 μg of cytosolic protein, per mixture. Tested reaction mixtures of total volume 100 μl were incubated in 2 ml-Eppendorf tubes at

37°C while shaking (450 RPM; Thermomixer Compact, Eppendorf). The reaction were stopped by adding 100 μl of acetonitrile and by rigorous vortexing. Reaction mixtures were then centrifuged at 13 000 RPM for 6.5 minutes (Centrifuge 5418, Eppendorf) and subsequently evaporated and dissolved, or used for analysis. Analysis was performed as previously described in Chapter 3.3.5.2.

3.3.5.4. *Study of the interaction of food supplements and sulfotransferases involved in biotransformation of xenobiotics*

The metabolism of food carcinogens and their interaction with food supplements was studied on rat liver and colon cytosols of control and pretreated animal models by the determination of SULT activity. Marker product, p-nitrophenyl sulfate, was separated and analyzed using optimized method RP-HPLC. The incubation mixtures (100 μl) were prepared in dublets, with the composition of reaction mixtures as follows:

- Rat liver/colon cytosol (protein content 2 mg/ml or 1 mg/ml, respectively)
- 100 μM PAPS (2 mM stock solution)
- 50 μM p-nitrophenol (2 mM stock solution)
- TRIS buffer (0.05 M Tris, pH 7.5, 15 mM $MgCl_2$)

All reagents were mixed in volumes to achive the above described concentration. The volume of 100 μl was attained by TRIS buffer. Immediately after iniciation of the reaction by PAPS, mixtures were gently vortexed and incubated 30 minutes at 37°C in open microtubes while shaking (450 RPM; Thermomixer Compact, Eppendorf). The reaction was stopped by an addition of 100 μl ice-cold acetonitrile. Then the mixture was vigorously vortexed and centrifuged at 13 000 RPM for 6.5 minutes (Centrifuge 5418, Eppendorf). The collected supernatant (190 μl) was evaporated in a vacuum evaporator (Centrivap Concentrator, Labconco, USA) to dryness and the residue was then dissolved in 30 μl of MilliQ water. An amount of 25 μl was taken for the RP-HPLC analysis, as described in Chapter 3.2.5.2.

4. RESULTS

4.1. Conjugation of peptides with a protein carrier

All five peptides (SULT 1B1, 1C1, 1E1, 2A1, 1C1/2) were well soluble in a coupling buffer and conjugated with mcKLH without forming clots. Peptides SULT1A1 and SULT1C2 had limited solubility, which was manifested as the formation of clots while dissolving. Additionally, they needed to be dissolved in dimethylsulfoxid not exceeding the limit content of 20%. Afterwards, all conjugates were prepared and used for chicken immunization.

4.2. Antibodies isolation and quantification

Collected eggs from control and treated hens were pooled into groups according to the immunization protocol. After the isolation process, the final volume of IgY fractions was determined and the protein concentration was evaluated by means of absorbance at 280 nm, using the previously mentioned formula (see Chapter 3.3.1.3). Due to the low affinity of several isolated IgY fractions towards peptide antigens (based on ELISA test, Chapter 4.3), immunization with rSULT1A1 and rSULT2A1 was repeated and new batches of fractions were isolated. Characterization of each fraction is shown in Tab. 6.

Table 6: Characterization of isolated IgY fractions.

IgY	Egg yolk [ml]	IgY fraction [ml]	c[mg/ml]	IgY yield [mg/ml of yolk]
Control	120	17	26.8	3.8
SULT1A1a	148	37	39.7	7.1
SULT1A1b	106	21	39.3	8.2
SULT1B1	220	34	43.3	6.7
SULT1C1	150	23	41.8	6.4
SULT1C2	231	35	44.8	6.8
SULT1E1	143	24	39.0	6.5
SULT2A1a	194	27	56.9	7.9
SULT2A1b	116	24	39.5	8.5
SULT1C1/2	186	23	48.4	6.0

a - first immunization batch, b- second immunization batch

4.3. Specificity of isolated antibodies

To verify the specificity of isolated antibodies, an ELISA test was performed. For the assay, all samples were prepared in duplicates, with a dilution of IgY antibody of 3.3,10, 30, and 90 μg/ml. Determined absorbance values at 405 nm, which correspond to IgY reactivity with antigens, are shown in Figure 7. Fractions of IgY SULT1A1a, 1B1, 1C1, 1E1, and 1C1/2 displayed a concentration dependent increase of absorbance, suggesting that the antibodies specifically recognized the peptide antigen. Some IgY fractions (SULT1A1b, 2A1, 2A1b), however, did not show sufficient reactivity with the respective antigen, which was manifested by very low absorbance values. The highest affinity, almost 2.5-fold higher, showed the IgY SULT1C1.

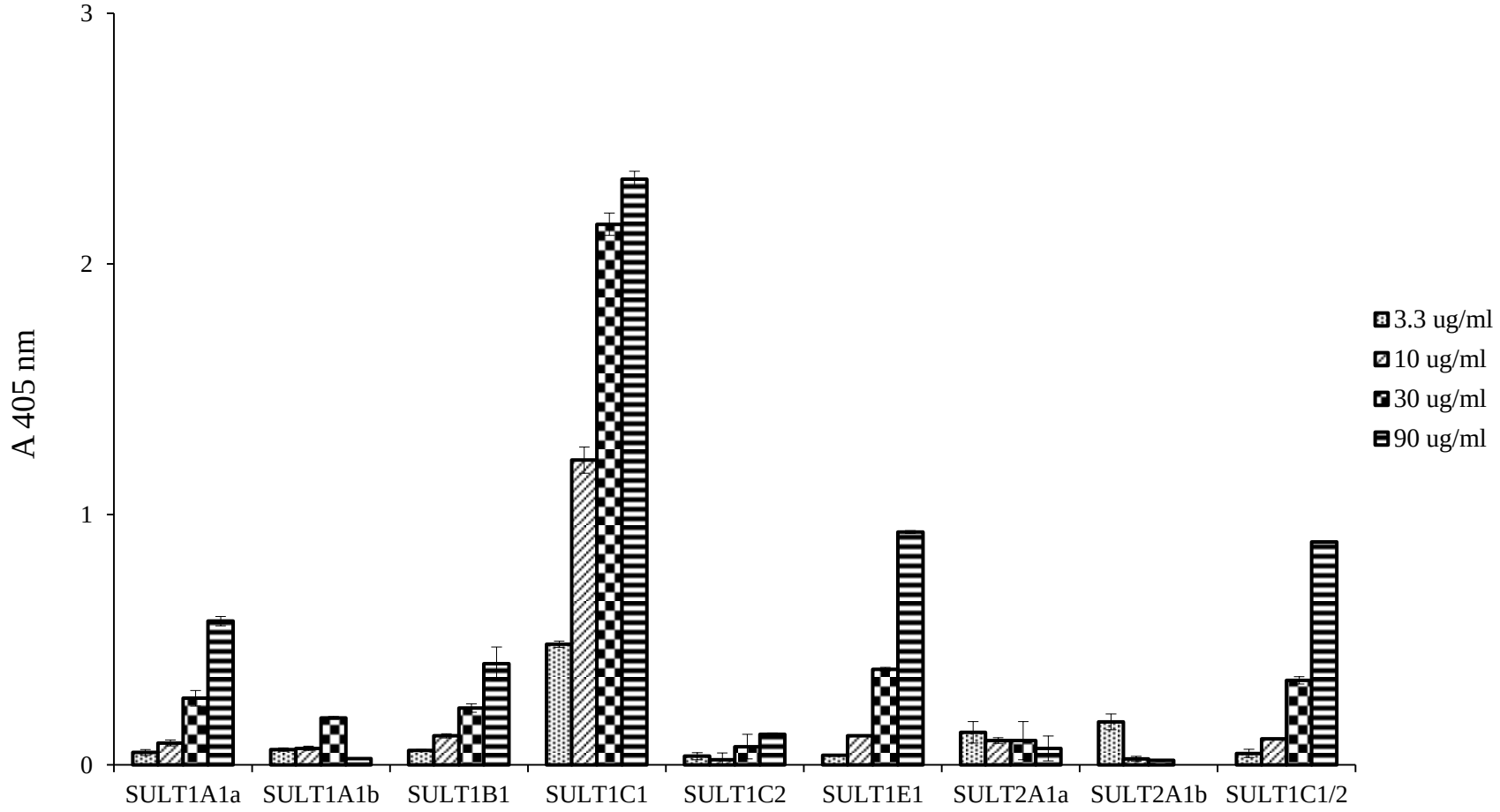


Figure 7: ELISA verification of the IgY fraction specificity. *Graph represents absorbance values at 405 nm of specific IgY fractions after control subtraction, at concentration 3.3, 10, 30, and 90 μg/ml. Data are means of duplicates with plotted ± SD. Value of SULT2A1b at 90 μg/ml was not plotted. a - first immunization batch, b- second immunization batch*

4.4. Affinity purification of anti-rat SULT1A1

In order to concentrate specific IgY and to avoid cross-reactivity, antibodies were affinity purified to be used for immunoblotting. Since SULT1A1 was of major interest, a 20 ml-IgY fraction was taken for the purification step. The efficiency has been evaluated by comparison of the absorbance at 405 nm of the original and purified IgY fraction (eluted by diethylamine, pH 11.5). The graph in Fig. 8 shows that there is at least 20-fold increase in the absorbance of specific antibody fraction in comparison to the original unpurified one, even at very low concentration. This result indicates that the procedure has been successfully conducted.

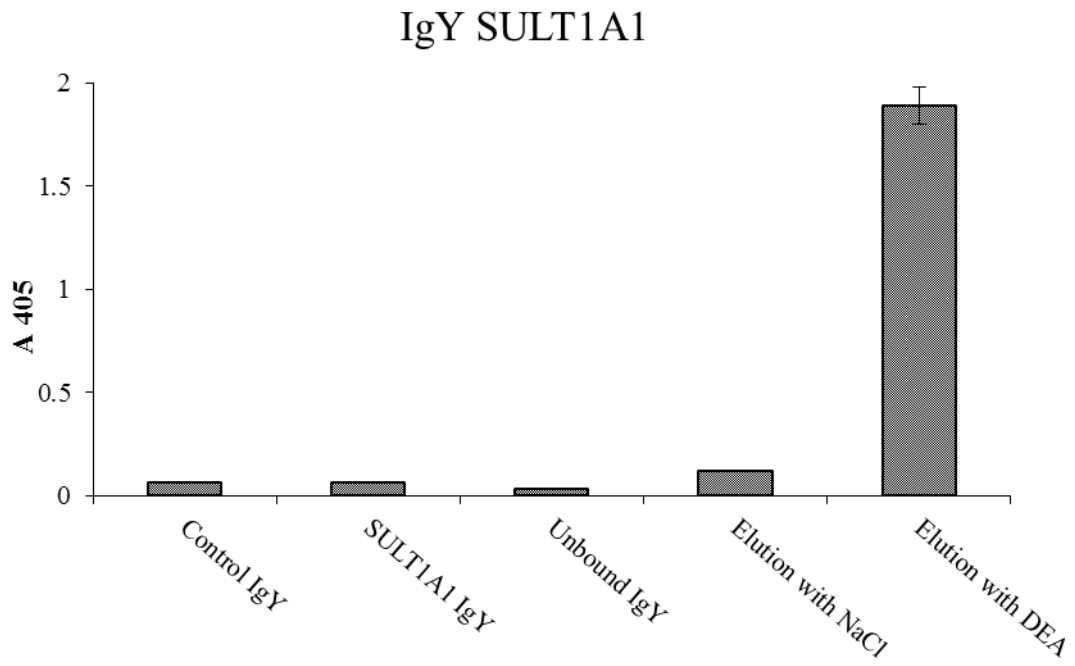


Figure 8: Evaluation of SULT1A1 affinity purification by ELISA. *IgY fractions were diluted to concentration 3.3 μg/ml.* ***Control IgY*** *= fraction of eggs collected before immunization;* ***SULT1A1 IgY*** *= original, unpurified antibody fraction;* ***Unbound IgY*** *= solution obtained after O/N incubation of antibody with peptide;* ***Elution with NaCl*** *=NaCl elution of weakly bound antibodies;* ***Elution with diethylamine*** *(DEA) =fraction of specific IgY, released by DEA*

In addition, the protein concentration and amount of IgY in each fraction was determined, using absorbance values at 280 nm (Tab.7). The purification step yielded 3.6 ml of specific antibody with protein concentration 343 μg/ml. In total, the amount of specific IgY represents around 0.15% of original SULT1A1 fraction.

Table 7: Protein concentration and amount of antibody in fractions obtained at purification step.

IgY fraction	Protein concentration [mg/ml]	Amount of IgY [mg]
SULT1A1 IgY	39.7	794.4
Unbound IgY	30.8	584.4
Elution by NaCl	1.1	13.1
Elution by DEA	0.3	1.2

4.5. Determination of protein concentration in rat cytosolic fractions

Rat liver and colon samples were characterized for protein concentration using the BCA protein assay. Liver samples originate from treated animals, which were administered by flavonoids (β-naphthoflavone, myricetin, dihydromyricetin) and/or the carcinogen compound PhIP. In addition, intestine samples of the same treated group were characterized for protein concentration as well (data not shown). Colon samples were isolated from rats treated with flavonoid compounds α-naphthoflavone, dihydromyricetin and/or carcinogen benzo[*a*]pyrene. For detailed administration schemes see *Appendix 1*. The protein concentration of liver cytosol and colon samples is shown in Table 8.

Table 8: Protein concentration of liver and colon cytosol fractions from rats, administered with chemopreventive compounds and/or carcinogens.

Liver cytosol	c [mg/ml]	Colon cytosol	c [mg/ml]
Control	26.1	Control	2.7
PhIP	22.2	ANF	1.9
BNF	38.2	BaP	1.9
BNF → PhIP	36.3	dHMR	1.7
MYR	37.1	ANF → BaP	2.9
dHMR	69.7	dHMR → BaP	1.3
		ANF + BaP	1.2
		dHMR + BaP	2.8

PhIP - 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; BNF - β-naphthoflavone; MYR - myricetin; dHMR - dihydromyricetin; ANF - α-naphthoflavone; BaP - benzo[*a*]pyrene

4.6. Effects of flavonoids and carcinogens on sulfotransferase protein level *in vivo*

The effects of tested flavonoid compounds and carcinogens on sulfotransferase protein level were assessed by the SDS polyacrylamide gel electrophoresis followed by Western blot technique. Proteins of rat liver cytosol (30 μg/well) and colon (15 μg/well) were resolved on 10% resolving gel and visualized by Coomassie Brilliant Blue dye (Fig. 9 and 10).

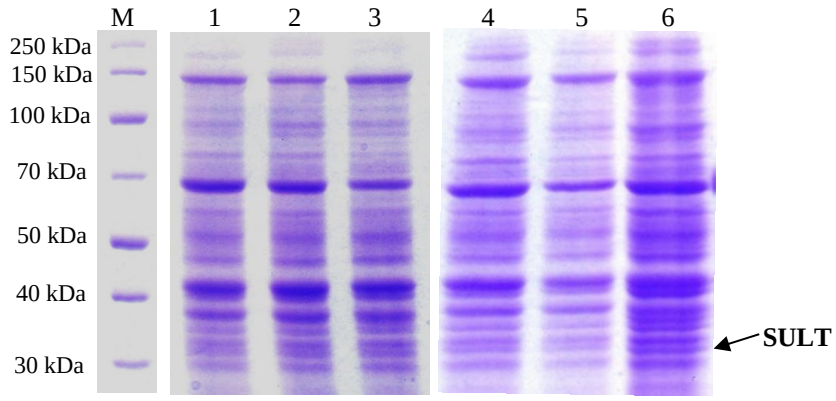


Figure 9: SDS-PAGE of rat liver cytosol. *Samples were separated on gel (3% stacking gel, 10% resolving gel) with protein concentration 1.5 mg/ml and volume 20 μl. In line M, 5μl of PageRuler Unstained Broad Range Protein Ladder (Thermo Scientific) was applied.*
***M** - marker; **1** - control; **2** - PhIP; **3** - BNF; **4** - BNF→PhIP; **5** - MYR; **6** - dHMR*
PhIP - 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; BNF - β-naphthoflavone;
MYR - myricetin; dHMR - dihydromyricetin

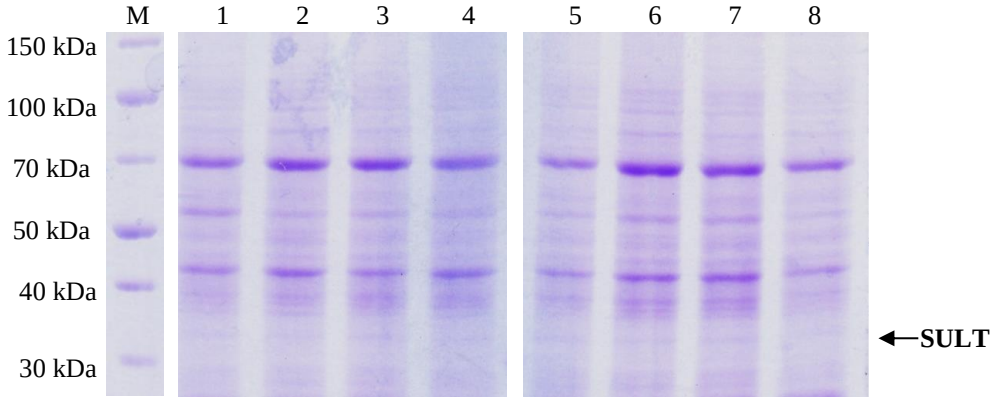


Figure 10: SDS-PAGE of rat colon cytosol. *Samples were separated on gel (3% stacking gel, 10% resolving gel) with protein concentration 0.75 mg/ml and volume 20 μl. In line M, 5μl of PageRuler Unstained Broad Range Protein Ladder (Thermo Scientific) was applied.*
***M** - marker; **1** - control; **2** - ANF; **3** - BaP; **4**- dHMR; **5** - ANF →BaP; **6** - dHMR→ BaP*
***7** - ANF + BaP; **8** - dHMR + BaP*
ANF - α-naphthoflavone; BaP - benzo[a]pyrene; dHMR - dihydromyricetin

Liver cytosol proteins (30 μg) separated by electrophoresis were transferred to a nitrocellulose membrane and detected using different isolated anti-rat chicken antibodies SULT1A1, 1B1, 1E1, 1C1, 1C2, and 2A1. The immunoblot analysis indicated very low specificity of antibodies, since there were none or only weakly visible bands developed beyond the expected position of SULT (blots not shown). Thus, the determination of xenobiotics effect on the SULT expression level was not possible and antibody purification was required.

First, the most abundant sulfotransferase 1A1 was purified (Chapter. 4.4). Rat liver and colon cytosol proteins (30/15 μg) separated by electrophoresis were transferred to a nitrocellulose membrane and subsequently detected using the affinity purified anti-rat SULT1A1 (concentration 3 μg/ml). Immunoblot analysis of rat liver cytosol showed no protein bands developed with specific anti-SULT 1A1 (blot shown in Fig. 11) as well as blot of colon cytosol samples (blot not shown). Thus, the determination of xenobiotics effects on sulfotransferases protein level was not possible.

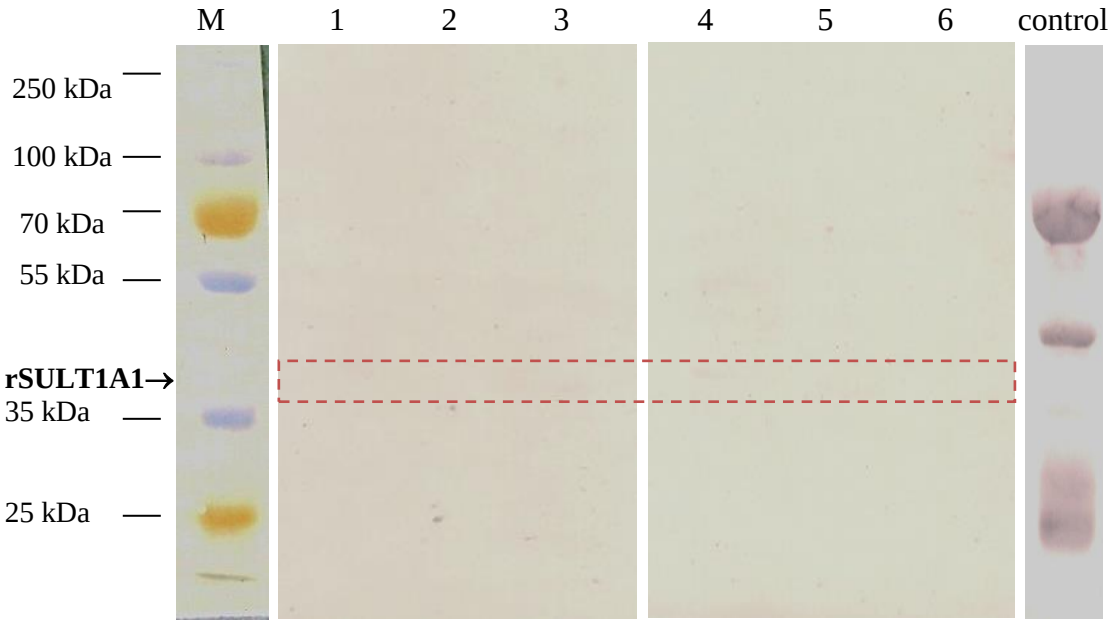


Figure 11: Western blot analysis of rat liver cytosol samples. *Electrophoresed cytosol proteins (30 μg) were transferred to membrane and probed with affinity purified chicken antibody against rSULT1A1 (c= 3 μg/ml). The arrow shows the expected postion of rSULT1A1.*
***M** - marker; **1** - control; **2** - PhIP; **3** - BNF; **4** - BNF→ PhIP; **5** - MYR; **6** - dHMR; **control** - positive control of 2'antibody*
PhIP - 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; BNF - β-naphthoflavone;
MYR - myricetin; dHMR - dihydromyricetin

4.7. Sulfotransferase activity assay

In this study, two assays were tested to determine the activity of sulfotransferases based on their own enzymatic reaction (Fig. 12). The first one was based on the detection of a PAP product obtained after conversion of PAPS (**1**; Chapter 4.7.1.) and the second one was based on the detection of the product p-nitrophenyl sulfate after reaction with substrate p-nitrophenol (**2**; Chapter 4.7.2.). Both compounds were separated using different HPLC systems and columns, and detected by their absorbance at 260 nm or 300 nm, respectively.

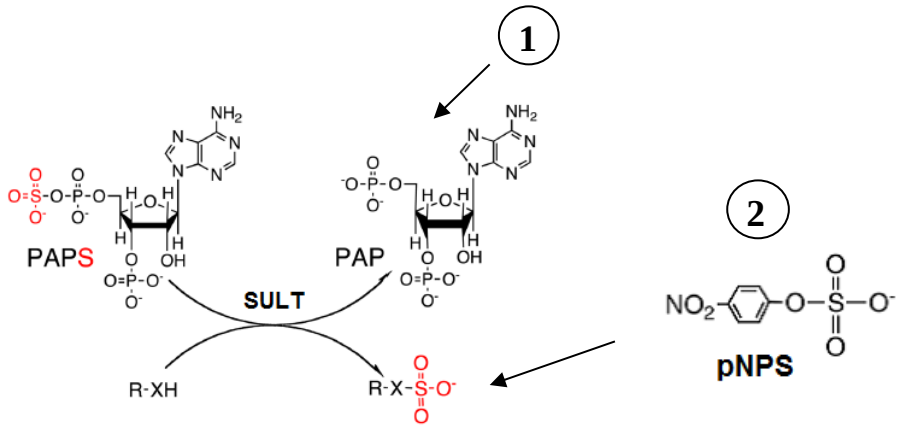


Figure 12: Sulfotransferase-catalysed transfer of SO_3^- from PAPS donor to R-XH acceptor.

4.7.1. Analysis of 3'-phosphoadenosine-5'-phosphate by High-performance liquid chromatography (HPLC)

One of the possibilities, how to determine sulfotransferase activity was to screen the conversion of PAPS to PAP, taking advantage of the fact that both compounds are nucleotides with similar structure and maximum absorbance at 260 nm. Therefore, the first goal was to separate both nucleotide standards on HPLC and determine their retention times (Fig. 13 and 14). Furthermore, analysis of PAPS confirmed that the commercially available PAPS contains significant amount of PAP, which was proved by the presence of a second peak of PAP (Fig 14, peak 1). The AUC value of PAPS represents about 65% of the AUC of both areas, therefore it fulfils the declared purity ≥ 60%. Repeated analysis confirmed the assumption that PAPS is also relatively an unstable compound, which decays in time to PAP.

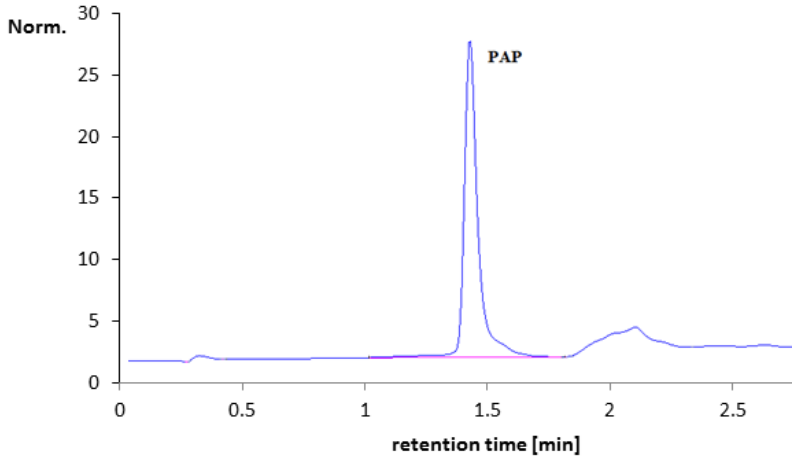


Figure 13: HPLC chromatogram of standard 3'-phosphoadenosine-5'-phosphate. *The retention time of 3'-phosphoadenosine-5'-phosphate (PAP) is 1.5 min. The analysis was conducted with detection at 260 nm. Injection volume 20 µl of 10 µM PAP. Flow rate 2ml/min.*

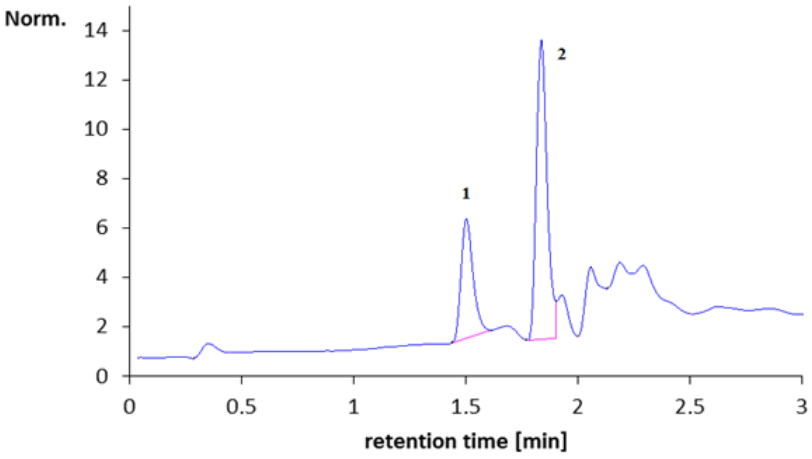


Figure 14: HPLC chromatogram of standard 3'-phosphoadenosine-5'-phosposulfate *The retention time of 3'-phosphoadenosine-5'-phosposulfate (PAPS) is 1.8 min.* ***Peak 1*** *= PAP;* ***Peak 2*** *= PAPS. The analysis was conducted with detection at 260 nm. Injection volume 20 µl of 20 µM PAPS.*

To find out if the analysis of sulfotransferase activity based on the determination of PAP is feasible, a test assay with a p-nitrophenol as a substrate was performed. In t = 0, the corresponding AUC values of peaks of PAP and PAPS were 48.9 and 67.5. While the AUC of PAP increased during the incubation reaction (t = 10 min → 57; t = 25 min → 58.5), the AUC value of PAPS firstly increased (t = 10 min → 71.2) and then decreased (t = 25 min → 68.1). Furthermore, a test SULT activity assay with the p-nitrophenol also showed, that the product of a sulfation reaction, p-nitrophenyl sulfate, which was separated under the same conditions has very similar retention time as the substrate p-nitrophenol (Fig. 15).

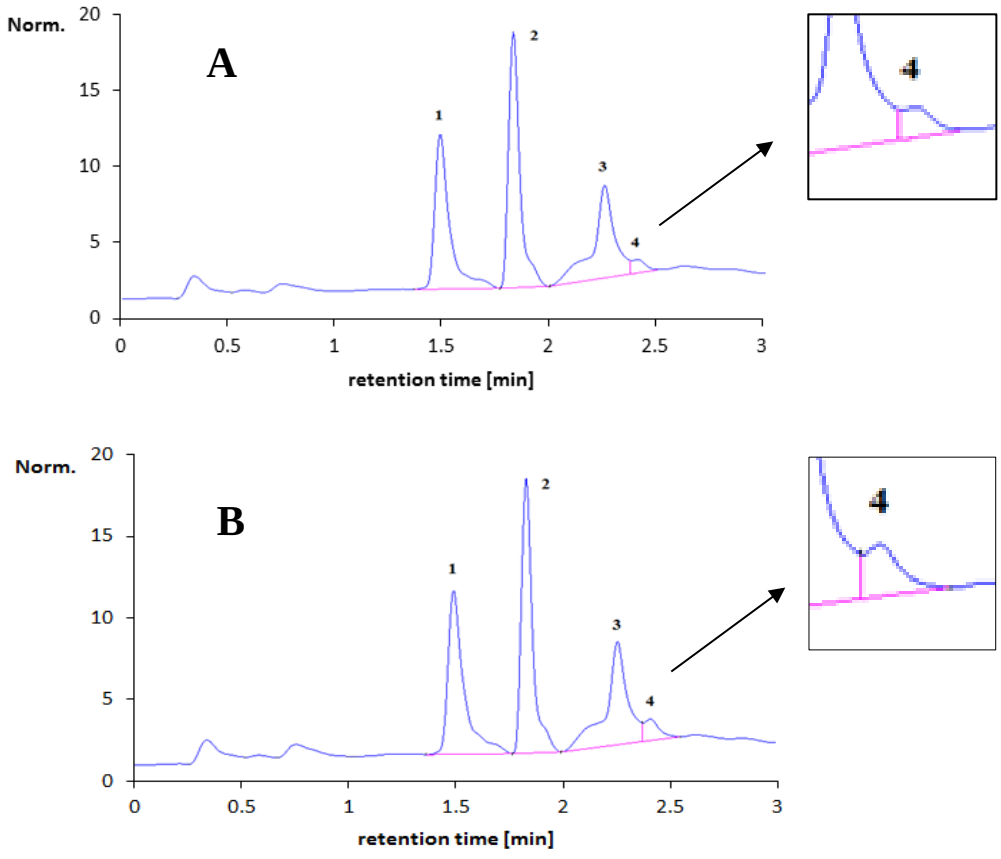


Figure 15: HPLC chromatogram of test incubation mixture. *An amount o 2 µg of SULT1A1 was incubated with substrate p- nitrophenol. The incubation time was 10 minutes (A) and 25 min (B), respectively. The amount of PAPS and PAP changed slightly. The resulting sulfoconjugate p-nitrophenyl sulfate is shown at position 4. Analysis was conducted with detection at 260 nm.*
***1** - PAP, **2** - PAPS, **3** - p-nitrophenol, **4**- p-nitrophenyl sulfate*

4.7.2. Optimization of sulfotransferase activity assay based on p-nitrophenyl determination

The second approach was to determine the sulfotransferase activity using p-nitrophenol as a probe substrate with different experimental conditions. The parental molecule and standard of generated conjugate p-nitrophenyl sulfate was separated using the RP-HPLC analysis and detected at UV absorbance 300 nm.

Optimization of assay conditions

The assay was optimized for incubation time, protein concentration, stopping solution, and concentration of sample for analysis. Furthermore, negative control assays were carried out (i) without protein, (ii) PAPS, and (iii) substrate to demonstrate assay reliability (data not shown). Based on several assays with different incubation times, 30 minute incubation was used, still in the linear region of the kinetics profile (data not shown). For stopping the reaction, acetonitrile and methanol were tested and acetonitrile was used for the analysis. Several different injection volumes were tested and the final procedure chosen was as follows: the reaction volume of incubation mixture (100 µl) was stopped by 100 µl of acetonitrile and subsequently reduced by evaporation to dryness. The residue was then dissolved in 30 µl of distilled water and 25 µl were used for the HPLC analysis. Based on results with different cytosol content, all assays were incubated with 200 µg per assay of liver cytosolic protein and 100 µg of colon cytosolic protein, since the protein concentration of colon samples was limited.

Separation and identification of p-nitrophenyl sulfate

Under experimental conditions (Chapter 3.2.5.2.), the retention time (RT) of p-nitrophenol was determined as about 6.61 minutes, while the RT for its conjugate is about 5.29 minutes (Fig.16). Additionally, p-nitrophenyl sulfate of known concentration ("spike") was added to the tested incubation mixture to prove the reliability of the assay (data not shown).

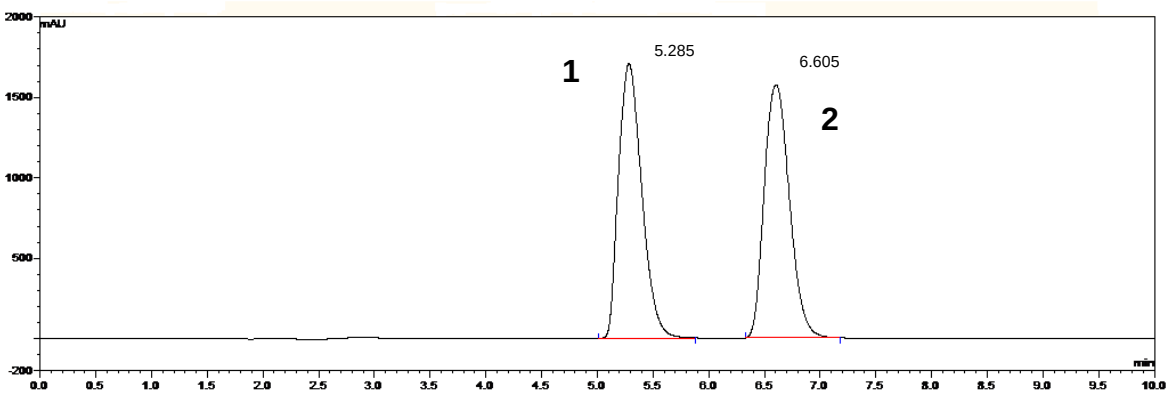


Figure 16: Chromatographic analysis of standard p-nitrophenol (peak 2) and p-nitrophenyl sulfate (peak 1). *The analysis was conducted with detection at 300 nm and injection volume 20 µl.*

Next, calibration curve has been constructed to calculate the amount of conjugate formed during the assays. Samples were prepared in duplicates and subsequently analysed on HPLC. The linearity of concentration of pNPS vs. obtained AUC values of pNPS is shown in Fig.17.

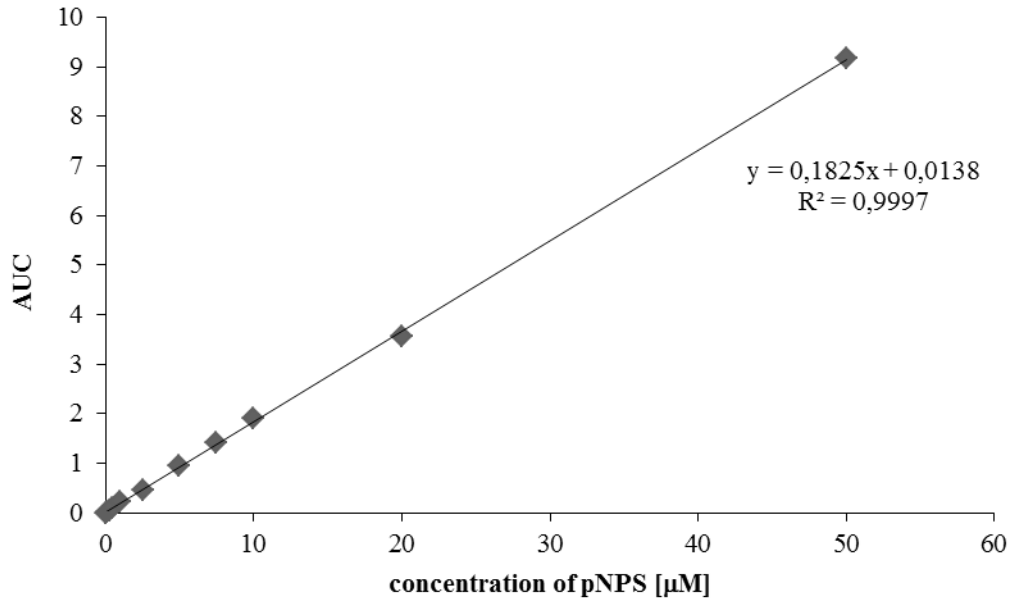


Figure 17: Calibration curve of p-nitrophenyl sulfate.
AUC - area under the curve; pNPS - p-nitrophenyl sulfate

4.7.3. Study of interaction of food supplements and sulfotransferases involved in biotransformation of xenobiotics

The effects of administered flavonoid compounds and carcinogens on the expression of sulfotransferases were assayed by means of the determination of their activity using the marker substrate p-nitrophenol. The separation and detection of its conjugate, p-nitrophenyl sulfate, was conducted using HPLC.

The activities of sulfotransferases in rat liver cytosol samples are shown in Fig.18. The highest activity of sulfotransferases was observed after PhIP treatment. Activity measured in cytosol samples of rats administered with combination of flavonoid compound BNF and carcinogen PhIP (BNF →PhIP), BNF, and MYR itself did not show higher increase in comparison to the control samples. The administration of dihydromyricetin caused only slight decrease in the activity of sulfotransferases.

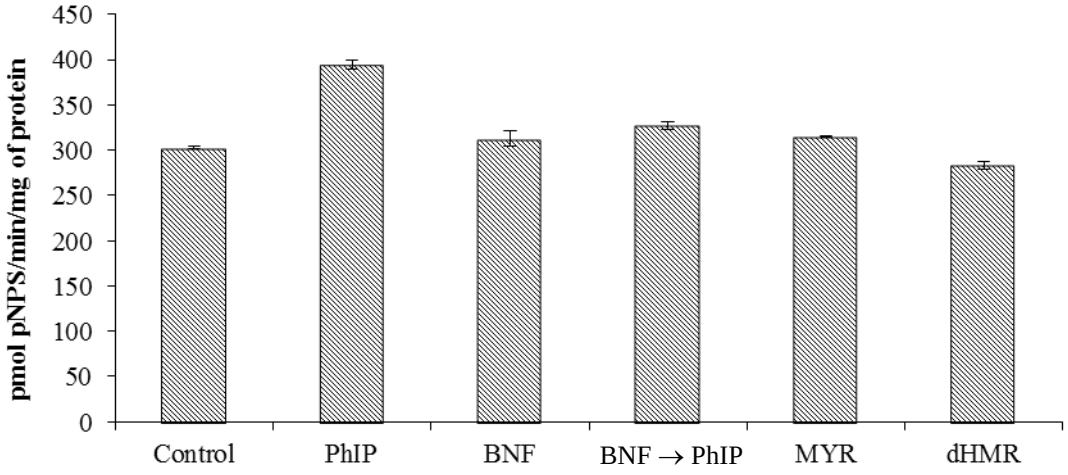


Figure 18: Effects of flavonoid compounds and/or carcinogens on the activity of sulfotransferases in liver cytosol samples. *The graph shows the average values of two assays with corresponding bars representing the means ± SD.*
pNPS - p-nitrophenyl sulfate; PhIP - 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; BNF - β-naphthoflavone; MYR - myricetin; dHMR - dihydromyricetin

The cytosolic fraction obtained from colon tissue has been characterized for sulfotransferase activity as well. The level of sulfation was very low and did not exceed the value of 10 pmol of pNPS/min/mg of protein (Fig. 19), which represents almost 100x lower activity than in liver tissue. The highest induction effect was observed after α-naphthoflavone administration. Interestingly, the activity after administration of α-naphthoflavone in combination with the carcinogen BaP was almost five times lower than

that after flavonoid treatment. The simultaneous administration of both compounds caused slightly lower activity of SULTs as well, but higher standard deviation was determined.

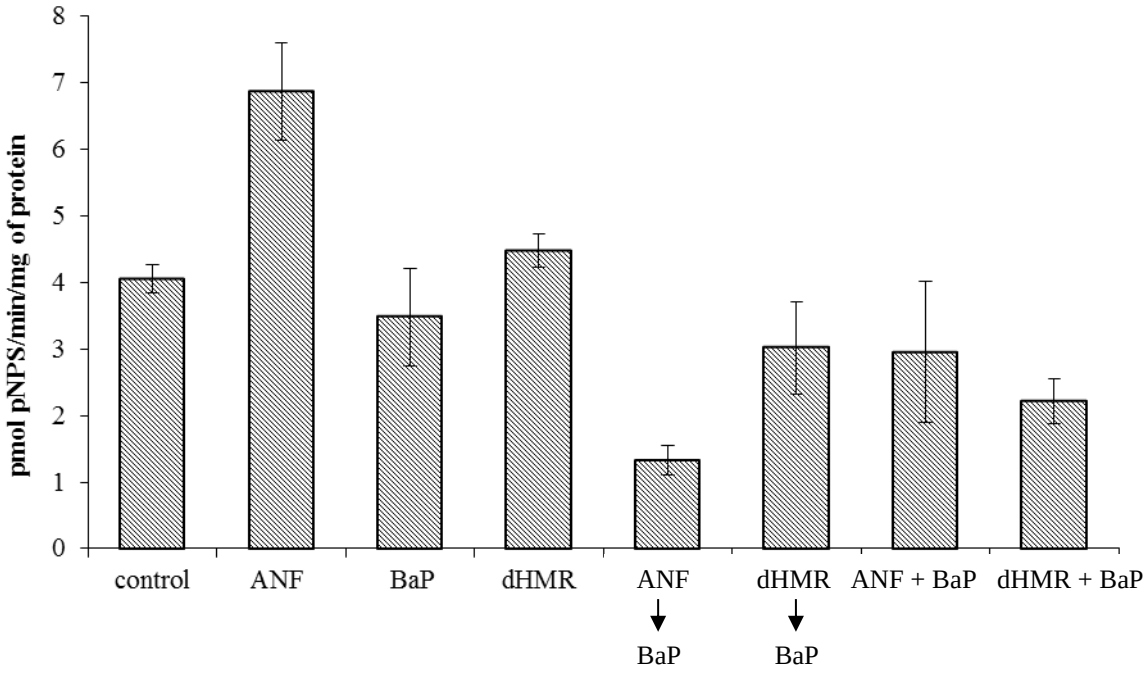


Figure 19: Effects of flavonoid compounds and/or carcinogens on the activity of sulfotransferases in colon cytosol samples. *The graph shows the average values of two assays with corresponding bars representing the ± SD.*
pNPS - p-nitrophenyl sulfate; ANF - α-naphthoflavone; BaP - benzo[a]pyrene; dHMR - dihydromyricetin

5. DISCUSSION

Cancer has become one of the leading diseases in the human population. However, carcinogenesis is a complex, multilevel process, which can be influenced at various stages by chemoprevention. Increasing attention is being paid to the chemopreventive compounds, especially phytochemicals, which are considered to have many beneficial effects on human health [98]. Thus, the use of dietary supplements containing phytochemicals has dramatically increased in the recent decade. However, high doses of such exogenous compounds might also negatively impact human health. Some enzymes of phase I and phase II of metabolism are responsible for procarcinogen bioactivation, and since they are mostly inducible, their expression and activity can be influenced by various effectors. Phytochemicals are known modulators of foreign compound metabolizing enzymes, therefore their induction may be associated with some possible adverse effects.

In this study, the effects of flavonoid compounds and carcinogens on phase II metabolism enzymes, sulfotransferases, has been investigated. The induction of sulfotransferases has been relatively less studied in comparison to other biotransformation enzymes. There are several *in vitro* studies on SULT induction based on the gene expression assessment using real-time RT-PCR methods [99,100]. Although the determination of induction based on the mRNA level is relatively easy, it may be misleading. Other assays should be required for the confirmation of results. The determination of SULT induction at the protein level provides more reliable data and thus has been conducted [30,31]. Furthermore, the activity of enzymes after xenobiotics treatment has been investigated in some studies as well [101], since the increased activity can promote procarcinogen activation and it is of interest to evaluate these effects as well. Therefore, the effects of flavonoid compounds and carcinogens on treated rats were investigated by Western blotting and optimized SULT activity assay.

To be able to analyse changes at protein expression level, it was necessary to have a suitable tool for the enzyme immunodetection. Specific antibodies capable of detecting rat sulfotransferases have limited commercial availability, therefore our own production was preferred. Instead of use of isolated or recombinant protein, synthetic peptide derived from the protein of interest may serve as an appropriate immunogen eliciting the required antibodies. The first goal of the study was to prepare peptide antigens derived from rat sulfotransferases - SULT1A1, 1B1, 1C1, 1C2, 1C1/2, 1E1, and 2A1. The conjugation of a

peptide with a protein carrier, KLH, is a critical step for further usage. Peptides SULT1A1 and SULT1C2 had limited solubility in buffer and required DMSO to be dissolved before their conjugation.

Recently, chicken egg yolk antibodies (IgY) are considered as an alternative to more frequently used mammalian ones. There are several advantages of this approach, as already reviewed in some publications [86,102] and briefly outlined in Chapter 1.5.4. Thus, hens were utilized to prepare anti-peptide antibodies for immunodetection of enzymes of interest. There are several approaches for IgY isolation [90], however, most of them require long-lasting steps such as ion-exchange chromatography or gel filtration. In this study, an isolation procedure based on freezing-thawing of the diluted yolks followed by sodium chloride precipitation at low pH was used, because the main advantage of this isolation procedure is its simplicity and cost-effectiveness. The application of this procedure results in antibodies of high purity. The yield of IgY was about 150 mg per yolk or 6.8 mg/ml of yolk, well corresponding to the usual antibody amount - approximately 100-150 mg per yolk [103].

To determine the specificity of isolated antibodies towards antigens and to evaluate the immunization process, an ELISA test using peptides as antigens was performed. Isolated antibodies were diluted to different concentrations by serial dilution (3.3, 10, 30 and 90 μg/ml), expecting that the signal would increase proportionally. Fractions of SULT 1A1a, 1C1, 1E1 and 1C1/2 showed an increasing tendency, while 1A1b, 1C2, 2A1a, and 2A1b did not show significant reactivity against the coated peptide antigen. The regular threefold increase is evident only in the fraction SULT1E1. The results of ELISA demonstrate the variability of the response of immune system to the peptide antigen or possible problems with immunogen preparation, which was later manifested in the Western blot analysis. Although the antibodies hardly react with the representative peptide antigen, it seems that the antibodies affinity towards the protein is either very small or none. It is known that despite the advantages of the peptide approach to develope antibodies, the ability of prepared anti-peptide antibodies to react with the respective proteins is about 30%.

In order to immunodetect specific proteins and avoid non-specific reactions and cross-reactivity with other antigens, purification of SULT1A1 IgY *via* affinity chromatography was performed. Enzyme SULT1A1 is the major sulfotransferase and also is the most abundant, therefore it has been further analysed. The results of affinity

chromatography were evaluated by ELISA (Fig.8, p.60) and the data indicate that the purification was successful. Furthermore, there is no evidence of remaining specific antibodies in other fractions, indicating that most of the specific antibodies were separated into the purified fraction. Although the specific antibodies represent approximately 0.15% of the total IgY fraction (average content is 1 - 10% of total antibodies [103]), they were successfully concentrated and prepared for further usage in the determination of flavonoid effects on SULT induction at protein level.

With the help of anti-peptide IgY antibodies, the effects of flavonoids and carcinogens have been investigated on rat models. Since flavonoids, as dietary supplements, are frequently consumed in human diet, their administration *via* gavage was performed to mimic human exposure (see *Appendix I*). For that purpose, rat liver cytosol samples were used, representing the main organ of biotransformation. Additionally, colon samples of treated rats were used as well. Figures 9 and 10 (p.62) show cytosol proteins after SDS polyacrylamide gel electrophoresis. The region of SULT, ≈ 33 kDa, shows bands possibly indicating the presence of sulfotransferases in the liver and colon cytosols in different amounts. However, anti-peptide antibodies did not specifically detect SULT enzymes on the membrane. Despite the fact that the purification of anti-peptide antibody against SULT1A1 was successful, the antibody did not detect these proteins on the membrane. Nevertheless, these results provide only preliminary insight and the induction effect of xenobiotics on SULT should be studied also with other SULT isoforms. For this purpose, it will be necessary to purify the rest of anti-peptide IgYs.

Because the immunological detection of changes at the protein level did not provide any results, it was important to determine the SULT activity. Several SULT activity assays have already been described in the literature. Most of them have been developed for phenolsulforansferases [104–106]. One of the approaches is based on the determination by the transfer of a PAP[^{35}S] sulfate group to different substrates, followed by a separation of the products by gel filtration [107], thin-layer or paper chromatography. Various non-radioactive endpoint methods have been developed as well [107,108]. They are usually based on special fluorometric properties of a unique substrate, colorimetric reactions or chromatograpical analysis by HPLC. Each method has its benefits (e.g. simplicity) and limitations (harmful radioactivity, time-consuming) that should be considered when selecting the optimal approach. This study focused on the optimization of sulfotransferase activity assay based on HPLC analysis and reaction with marker substrate, therefore two

assays were tested to determine the activity of sulfotransferases based on the their own enzymatic reaction. Probe substrates specific for individual members of the SULT family, especially for SULT1A1, are discussed and evaluated in many studies [110,111]. It has been proved that sulfotransferases have a broad substrate specifity and most of them are expressed in various levels in different tissues. Therefore it is of interest to note that the tissue cytosol samples are usually a complex mixture of different isoenzymes, some or all of which may have some activity toward the probe substrate. Thus, it is quite difficult to determine the contribution of each enzyme.

The first approach was to analyse the product of sulfatation reaction, PAP, as a marker of sulfotransferase activity. Standards of nucleotides (PAPS, PAP) were separated under given conditions and retention times were determined. However, the analysis on HPLC shows that PAPS contain significant amount of PAP (Fig.14, p.65) and additionally, PAPS spontaneously converts to PAP with time (when not frozen). It is therefore not possible to use the PAP determination as a measure of SULT activity. We thus considered to perform a test SULT activity assay with the p-nitrophenol as a marker substrate. The product of a sulfation reaction, p-nitrophenyl sulfate, was separated under the same conditions, however, it has very similar retention time as the substrate p-nitrophenol thus the separation and analysis is also not applicable.

The second approach, how to determine the sulfotransferase activity in rat liver and colon cytosol samples, was based on the HPLC determination of sulfation product formed from the marker substrate, p-nitrophenol. The enzyme of interest, SULT1A1, is known to be the most important sulfotransferase in the xenobiotic metabolism, since it is the major SULT expressed in adult human liver. It has the lowest K_m value for p-nitrophenol among the other sulfotransferases, however, it is not the only one capable of sulfatation. Substrate p-nitrophenol is also metabolised by SULT1B1 or SULT1A3, but with significantly higher K_m values. Despite these facts, pNP still remains one of the most widely used probe substrates in many studies. Hence, we used this substrate and adopted it for our cytosolic system. Within the optimization process, the optimal assay conditions were identified and a calibration curve was used to determine the amount of pNPS formed during the conjugation reaction.

Based on the optimized assay for the determination of SULT activity the effect of administered food supplements and carcinogens on sulfotransferases was assessed. The activity of sulfotransferases among assayed rat liver cytosols showed only small changes,

compared to control cytosols. The only high SULT activity in liver cytosol was observed after PhIP treatment, while the combination of a flavonoid compound and a carcinogen in sequence administration caused only slight activity potentiation. It has been shown that β-naphthoflavone has a slight induction effect on sulfotransferase SULT1A3 in Hep G2 cells [74], thus this isoenzyme may have contributed to this increase in activity in BNF-pretreated rats. The SULT activity in colon cytosols was approximately 100x lower in comparison to liver and did not exceed 10 pmol/min/mg of protein. This is probably because the liver is the main detoxication organ and the level of enzymes present is significantly higher than in the colon. The highest activity in colon cytosol samples was observed after α-naphthoflavone treatment, while the lowest activity was observed after sequential administration of α-naphthoflavone and benzo[*a*]pyrene. The other changes of activities are insignificant, since they are generally low.

Taking together, a further determinations of SULT activity in different tissues need to be performed to evaluate the SULT induction by various xenobiotic compounds in more details. Furthermore, similarly to the liver, the small intestine is well equipped with various sulfotransferases, which contribute to the biotransformation of xenobiotics as well, since it is the major site of their absorption.

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6. SUMMARY

This study focused on phase II metabolism enzymes, sulfotransferases, which are involved in some cases in the biotransformation of carcinogens. The induction of sulfotransferases by various chemopreventive compounds is an important feature and has not been yet well studied, therefore this represents an important field of study to be contribute to.

The following tasks were performed and findings were made as summarized below:

- Chicken antibodies (IgY) against selected peptides derived from rat sulfotransferases SULT1A1, 1B1, 1C1, 1C2, 1C1/2, 1E1, and 2A1 were prepared and anti-peptide SULT1A1 IgY was successfully purified by affinity chromatography.
- The anti-peptide IgY, however, failed in detection of SULT1A1 at the protein level in rat liver cytosols.
- Two SULT activity assays employing HPLC were tested - one based on the determination of PAP and the second one on the detection of p-nitrophenyl sulfate. The second optimized approach was utilized for further studies.
- SULT activities in rat liver cytosol, with the exception of PhIP administration, did not show marked changes in response to animal treatment. Solely the PhIP administration evidently increased SULT activity.
- The SULT activities determined in colon cytosols were approximately by 100x lower in comparison to liver and did not significantly change after animal treatment

7. REFERENCES

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Appendix I.

Treatment

Rat liver cytosol samples

Regimen I

Flavonoids (myricetin, dihydromyricetin), dissolved in sunflower oil (0.5 ml) were *p.o* administered by gastric gavage 60 mg/kg body weight, daily for 4 consecutive days.

The control group was treated with 0.5 ml of sunflower oil.

The treated rats were fasted overnight and next day (24 hours after the last treatment) were sacrificed.

Regimen II

β-Naphthoflavone was dissolved in sunflower oil (0.5 ml) and *p.o* ββadministered in a concentration of 60 mg/kg b.w. After 72h, one dose of PhIP (50 mg/kg b.w.) was administered. The treated rats were fasted overnight and next day (24 hours after the last treatment) were sacrificed.

Each compound was also administered separately. One dose of β-naphthoflavone was administered in a concentration of 60 mg/kg b.w. The rats were sacrificed 96h after the treatment. One dose of PhIP was administered in a concentration of 50 mg/kg b.w. The rats were sacrificed 24h after the treatment.

The scheme of treatment is shown in Table 1.

Table 1: **Scheme of administration**

	Regimens					
Groups	**Control**	**PhIP**	**BNF**	**BNF → PhIP**	**MYR**	**dHMR**
Day 1	SO	-	BNF	BNF	MYR	dHMR
Day 2	SO	-	-	-	MYR	dHMR
Day 3	SO	-	-	-	MYR	dHMR
Day 4	SO	PhIP	-	PhIP	MYR	dHMR
Day 5	sacrifice					

SO = sunflower oil; BNF - β-naphthoflavone; PhIP - 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; MYR - myricetin, dHMR - dihydromyricetin

Rat colon cytosol samples

The control group was treated with 0.5 ml of sunflower oil.

All tested compounds were dissolved in sunflower oil.

Regimen I

The tested chemopreventive compounds α-naphthoflavone or dihydromyricetin were administered by gastric gavage (120 mg/kg body weight) to animals. The treated rats were sacrificed 96h after treatment.

Regimen II

Flavonoids α-naphthoflavone or dihydromyricetin were administered in concentration of 120 mg/kg b.w. 72h after, one dose of carcinogen benzo[*a*]pyrene (120 mg/kg b.w.) was administered to both groups. The treated rats were fasted overnight and 24h after the last treatment, the animals were sacrificed.

Regimen III

Carcinogen benzo[*a*]pyrene was administered in dose of 120 mg/kg b.w and after 24h the treated animals were sacrificed.

Regimen IV

Flavonoids α-naphthoflavone or dihydromyricetin were administered simultaneously with carcinogen benzo[*a*]pyrene (all 120 mg/kg b.w) and the rats were sacrificed 24h after treatment.

The scheme of treatment is shown in Table 2.

Table 2: **Scheme of administration**

	Regimens							
Groups	**control**	**ANF**	**dHMR**	**BaP**	**ANF→BaP**	**dHMR→BaP**	**BaP + ANF**	**BaP + dHMR**
Day 1	SO	ANF	dHMR	SO	ANF	dHMR	SO	SO
Day 2	-	-	-	-	-	-	-	-
Day 3	-	-	-	-	-	-	-	-
Day 4	SO	SO	SO	BaP	BaP	BaP	BaP + ANF	BaP + dHMR
Day 5	sacrifice							

SO = sunflower oil; ANF - α-naphthoflavone; dHMR - dihydromyricetin; BaP - benzo[*a*]pyrene,

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